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**14. ABSTRACT**

The long term goals of this research are to understand the mechanisms by whichNF1 and its partners control growth using the Drosophila peripheral nerve as our assay system. This system is advantageous because we can apply a number of powerful molecular genetic methodologies that are not available in other systems. Our major findings were generated from aim #4. We reported after year2 that Ras nonautonomously activates perineurial glial growth via PI3 Kinase, Akt and FOXO (published in the Journal of Neuroscience in 2007). Second, we report preliminary evidence that pushover (push) acts in the glia to regulate perineurial glial growth, but in the neuron to regulate excitability. Third, we report that activation of the Tor pathway is necessary but not sufficient to increase perineurial glial growth. Fourth, we report that the metabotropicglutamate receptor regulates neuronal growth and excitability via PI3K.

**15. SUBJECT TERMS**

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## INTRODUCTION

Over the last several years, my lab has been developing the Drosophila peripheral nerve as a system with which to identify and study the signalling pathways controlling growth of the perineurial (outer) glial layer. To accomplish this goal, we apply the various molecular genetic methodologies uniquely available in Drosophila; we hope that these methodologies will enable us ultimately to identify all of the relevant genes that interact with *NF1* to control growth, and place *NF1* and these partner genes in as complete a mechanistic context as possible. Then this mechanism could be tested and refined in systems more similar to humans but more difficult to work with (e.g. the mouse). Because all of the experiments are performed on the acutely dissected third instar larva, there are no complications or caveats associated with experimentation on cell culture systems, and we assay the entire nerve cross section as it exists within the whole organism. We thought that a more complete mechanistic understanding of growth control within peripheral nerves would greatly facilitate the ability to design drugs able to combat neurofibromas. Within this larger context, I proposed three different tasks to investigate various aspects of the genetic control of growth within peripheral nerves. These tasks involve elucidation of the relationship among Neurofibromin, pushover, and protein kinase A, as well as the identification of signalling pathways downstream of Ras that affect growth and neuronal excitability within peripheral nerves. For task #2, we have collected data demonstrating that Pushover (push) acts in the motor neuron to control motor neuron excitability, but in preliminary data appears to act in the glia to control perineurial glial growth. We report the most success from our experiments with task #3, in which we demonstrated that Ras activates perineurial glial growth via PI3K, Akt and Foxo, and in preliminary findings we also collected data suggesting that the Tor pathway cooperates with Foxo in regulating glial growth, and that the EGF ligand spitz might participate in glial growth control. We also report that ligand activation of the single Drosophila metabotropic glutamate receptor DmGluRA activates PI3K in motor nerve terminals, and activated PI3K, in turn, promotes neuronal growth and arborization via the Tor/S6 Kinase pathway, and decreases neuronal excitability via inhibition of the transcription factor Foxo.

## BODY

<u>Task one (completed): Does Neurofibromin activate PKA?</u> Several experiments were proposed in the grant application to address this possibility, and many of these were completed during the first year of funding. However, as discussed in the first year report, the completed experiments gave inconclusive and in some cases conflicting results, making it impossible to place the data in a mechanistic framework. For example, during year 1, I proposed to determine: first, if expression of $NF1^{+}$ specifically in peripheral glia suppressed the effects of the null mutation $NF1^{P2}$ (The et al., 1997) on perineurial glial growth, second: test if overexpression of *NF1* in peripheral glia enhanced the effects of the constitutively active $\mathrm{Ras}^{\mathrm{V12}}$ (Lee et al., 1996) on perineurial glial growth, third: test if loss of function mutations in *PKA* suppress the effects of $\mathrm{Ras}^{\mathrm{V12}}$ on perineurial glial growth, and fourth: test the prediction that the constitutively active *PKA* called *PKA-C1** (Li et al., 1996) is epistatic to $NF1^{P2}$ in its interaction with $Ras^{V12}$. The results of these experiments are summarized in Figure 1 and answers to each specific question are described below.

<u>Does expression of *NF1* specifically in the peripheral glia suppress the effects of $NF1^{P2}$ on perineurial glial growth?</u> Expression of $Ras^{V12}$ in peripheral glia thickens perineurial glia, and this effect is suppressed by the $NF1^{P2}$ mutation. To determine if lack of *NF1* specifically within the peripheral glia was responsible for this suppression, we introduced a *UAS-NF1* transgene (kindly supplied by Andre Bernards) into larvae carrying both *gli-GAL4*, in which the *Gal4* transcription factor is expressed specifically in peripheral glia (Sepp et al., 1999) and $UAS\text{-}Ras^{V12}$, all in the presence of the $NF1^{P2}$ mutation. In these larvae, wildtype *NF1* would be expressed only in the peripheral glia; the rest of the larval cells would remain mutant for *NF1*. We found that, indeed, expression of $NF1^{+}$ in the peripheral glia rescued this mutant effect of $NF1^{P2}$. In particular, perineurial glial thickness was increased from 1.7 µm in the absence of *UAS-NF1*, to 2.6 µm in the presence of *UAS-NF1* (compare lanes 2 and 3, figure 1 below). These results suggest that loss of *NF1* specifically in the peripheral glia causes the suppression of the effects of $\mathrm{Ras}^{\mathrm{V12}}$.

<u>Does overexpression of *NF1* specifically in the peripheral glia enhance the effects of $\mathrm{Ras}^{\mathrm{V12}}$ on perineurial glial growth?</u> To address this question, we co-expressed $Ras^{V12}$ and *NF1* in peripheral glia, and compared perineurial glial thickness to larvae expressing $Ras^{V12}$ alone. We found that there was no significant difference in perineurial glial thickness between the two genotypes (compare lanes #1 and #4, Figure 1 below), suggesting that our hypothesis was incorrect: overexpressing *NF1* in peripheral glia does not enhance the effects of $Ras^{V12}$.

<u>Does reduction in PKA activity suppress the effects of Ras$^{V12}$ on perinurial glial growth?</u> Expression of a constitutively active PKA enhances the effects of Ras$^{V12}$, Neurofibromin activates PKA (Tong et al., 2002), and the *NF1$^{P2}$* mutation suppresses the effects of Ras$^{V12}$. These observations led to the prediction that reduction in PKA activity would suppress the effects of Ras$^{V12}$. To address this question, we measured perineurial glial thickness in larvae expressing Ras$^{V12}$ and heterozygous for a *PKA* null mutation (*PKA$^{H2}$*). This allele is expected to reduce PKA activity in the larva by 50%. We found no effect on perineurial glial growth (compare lanes #1 and #5 in Figure 1, below). Then we replaced the *PKA$^{H2}$* allele for this experiment with two alleles expected to reduce PKA activity: a deletion of *PKA* called gamma-15, which is expected to reduce PKA activity by 50%, as well as a transgene expressing a dominant-negative PKA allele called *BGO*: this transgene is a mutant form of the PKA regulatory subunit which fails to bind cAMP, and thus constitutively represses the endogenous, wildtype PKA. Again, we found no effect of this reduction in PKA activity on perineurial glial growth (compare lanes #1 and #6 in Figure 1 below).

Figure 1: Effects of altered *NF1* activity on perineurial glial growth in larvae expressing Ras$^{V12}$

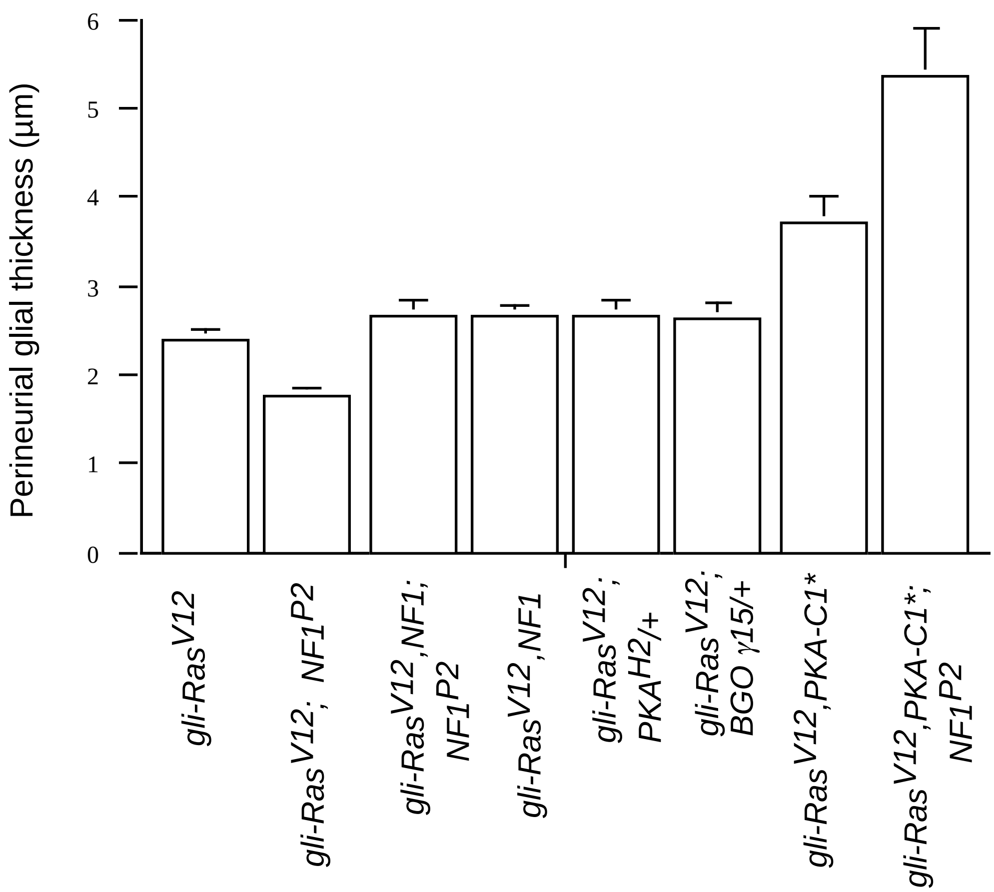


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Figure 1: Mean perineurial glial thickness (µm, +/- SEM) is shown along the Y axis for the genotypes indicated along the X axis. The following pairwise combinations had statistically significant differences (Student's unpaired t-test): For *gli-Ras$^{V12}$; NF1$^{P2}$* (lane #2), n= 41 vs. *gli-Ras$^{V12}$-NF1; NF1$^{P2}$*(lane #3), n=30, $p < 0.0001$; for *gli-Ras$^{V12}$,PKA-C1*; NF1$^{P2}$*(lane#8), n=16 vs. *gli-Ras$^{V12}$; NF1$^{P2}$* (lane #2), n=41, $p < 0.0001$. For *gli-Ras$^{V12}$,PKA-C1*(lane #7) n=41 vs. *gli-Ras$^{V12}$,PKA-C1*; NF1$^{P2}$*(lane#8), n=16, p=0.0056.

<u>Is expression of the constitutively active PKA epistatic to *NF1$^{P2}$* in its interaction with Ras$^{V12}$?</u> *NF1$^{P2}$* suppresses the effects of Ras$^{V12}$, whereas expression of the constitutively active PKA (called PKA-C1*) enhances the

effects of $Ras^{V12}$. If $NF1^{P2}$ exerts its suppression by reducing [cAMP] and thus PKA activity, then expression of PKA-C1* is predicted to be epistatic to $NF1^{P2}$ because PKA-C1* does not require cAMP for activity. We tested this possibility by co-expressing $Ras^{V12}$ and PKA-C1*in peripheral glia in an $NF1^{P2}$mutant background. We found that, as predicted, *PKA-C1** was epistatic to $NF1^{P2}$: The perineurial glia, in an $NF1^{P2}$ background and in the presence of both $Ras^{V12}$ and PKA-C1* was extremely thick, actually even thicker than in an $NF1^{+}$ background (compare lanes#2, #7 and #8 in Figure 1).

Overall conclusions, Task one: Our observation that overexpression of *NF1* in peripheral glia has no effect on perineurial glial growth is not inconsistent with the mechanisms proposed in the grant application. This observation probably means that normally, Neurofibromin levels are not limiting for the signalling pathways in which it operates; thus, overexpression is phenotypically silent. The inability of reductions of PKA to suppress the effects of $Ras^{V12}$ is not consistent with the central hypothesis. One possibility is that we are unable to lower PKA activity sufficiently to observe the anticipated suppression of the effects of $Ras^{V12}$. Unlike *NF1*, *PKA-C1* is an essential gene, so a reduction of PKA to zero kills Drosophila prior to the third instar larval stage that we assay. In this view, we are unable to lower PKA activity sufficiently to confer suppression and still retain viability. An alternative possibility, of course, is that Neurofibromin does not act through PKA.

The ability of *NF1* to rescue $NF1^{P2}$ when expressed only in the peripheral glia is consistent with our central hypothesis, as is the demonstration that *PKA-C1** is epistatic to $NF1^{P2}$. However, I have noticed that there is a lot of larva to larva variability associated with *NF1* mutations which makes me want to move cautiously in reporting these results. It is possible that some of this variability reflects genetic background effects; such effects are also observed with *NF1* mutations when the small size phenotype is assayed. To address this possibility, in year two we isogenized the transgenes and mutations listed in this report by back-crossing five times to our isogenic wildtype strain. Then we re-tested the effects of the $NF1^{P2}$ null mutation on perineurial glial growth in larvae expressing $Ras^{V12}$ in the peripheral glia, but this time used stocks in which each genetic element was isogenized (by five successive back-crosses) to our isogenic wildtype stock. We found that in an isogenic genetic background, the $NF1^{P2}$ mutation actually enhanced, rather than suppressed, the growth promoting effects of $Ras^{V12}$. In particular, perineurial glial thickness in $NF1^{P2}$; *gli-*$Ras^{V12}$ larvae was 3.1 +/- 0.2 µm (n=13), which is significantly thicker than glial thickness in $NF1^{+}$*; gli>*$Ras^{V12}$ (2.2 +/- 0.1). This unexpected result is definitive, as this result controls for genetic background effects. The enhancement of $Ras^{V12}$ by $NF1^{P2}$ could be the result of loss of *NF1* in the perineurial glia, or the peripheral glia. We planned to distinguish between these possibilities by determining if expression of $NF1^{+}$ specifically in the peripheral glia was able to rescue this phenotype. All of the necessary mutations and transgenes were isogenized and the necessary stocks constructed to perform this experiment. Unfortunately, we were unable to obtain any larvae homozygous for the $NF1^{P2}$ mutation so we were unable to make the desired measurements. I don't think this lack of larvae results from any interesting genetic interaction - rather, I think that combining the generally debilitating $NF1^{P2}$ mutation with the simultaneous presence of three additional transgenes decreased larval viability to below a frequency needed to observe any larvae in a realistic number of bottles.

Task two: A key question in this task was an identification of the cell type in which Pushover (Push) functions to control perineurial glial growth (Yager et al., 2001). The three possible cell types are: the neuron, the peripheral glia, and the perineurial glia. We have approached this question in two ways, first by identifying the cell type in which Push must function for proper control of perineurial glial growth, and second, by identifying the cell type(s) in which Push protein is present. These two approaches provide complementary information, and both approaches together provide a believable answer to the question posed. We have made good progress on both fronts.

*First, to identify the cell type in which Push must function*: we constructed a *push* RNAi construct under *UAS* control and obtained transgenic flies carrying this construct. To test for the ability of this RNAi construct to knockdown *push* levels sufficiently to observe a phenotype, we assayed for the *push* motor neuron excitability defect, which is monitored with electrophysiology moethds. This defect is more robust and easier to observe than the perineurial glial phenotype. We drove *push-RNAi* with the *Gal4* driver *D42*, which expresses in motor neurons, and *gli-Gal4*, which expresses in peripheral glia. We found that when both driver and *push-RNAi* were heterozygous, we could observe no electrophysiological phenotype with either driver. However, when both the *D42* drives and *push-RNAi* were homozygous, then we observed an increase in neuronal excitability similar to, but not as extreme, as the *push* null mutant. In contrast, larvae homozygous for *gli>push-RNAi* exhibited wildtype neuronal excitability (not shown). These experiments demonstrate that the *push-RNAi* construct can knockdown *push* activity but only when homozygous, and this construct may not generate a complete null phenotype. These experiments also demonstrate that the *push* mutations increase neuronal excitability because of lack of *push* in neurons, not glia.

To identify the cell type within which *push* functions to control perineurial glial thickness, we drove *push-RNAi* with each of three *Gal4* drivers: *D42*, *gli-Gal4*, and a third driver, *repo-Gal4,* which expresses in both peripheral and perineurial glia. We found that in larvae heterozygous for *D42>push-RNAi*, and *repo>push-RNAi*, perineurial glial thickness was normal, whereas in *gli>push-RNAi*, perineurial glial thickness was significantly, although moderately, elevated. Given that the chromosomal *push* null mutation confers only a moderate increase in perineurial glial thickness in an otherwise wildtype background, these preliminary findings are consistent with the notion that *push* acts in the peripheral glia to control perineurial glial thickness, as hypothesized. The inability of *repo-Gal4* to confer a perineurial glial phenotype when driving *push-RNAi* might result from inadequate knockdown of *push* levels in these larvae heterozygous for each transgene, as described above for the motor neuron excitability phenotype. If so, then perhaps doubling the dosage of the *push-RNAi* transgene might knock down *push* levels further, and to a level sufficient to observe a perineurial glial thickness phenotype. To test this possibility, we assayed perineurial glial thickness in larvae carrying one copy of *repo-Gal4* and homozygous (two copies) of *UAS-push-RNAi*. We found an even greater increase in perineurial glial thickness in these larvae, which is consistent with a role for *push* in the glia.

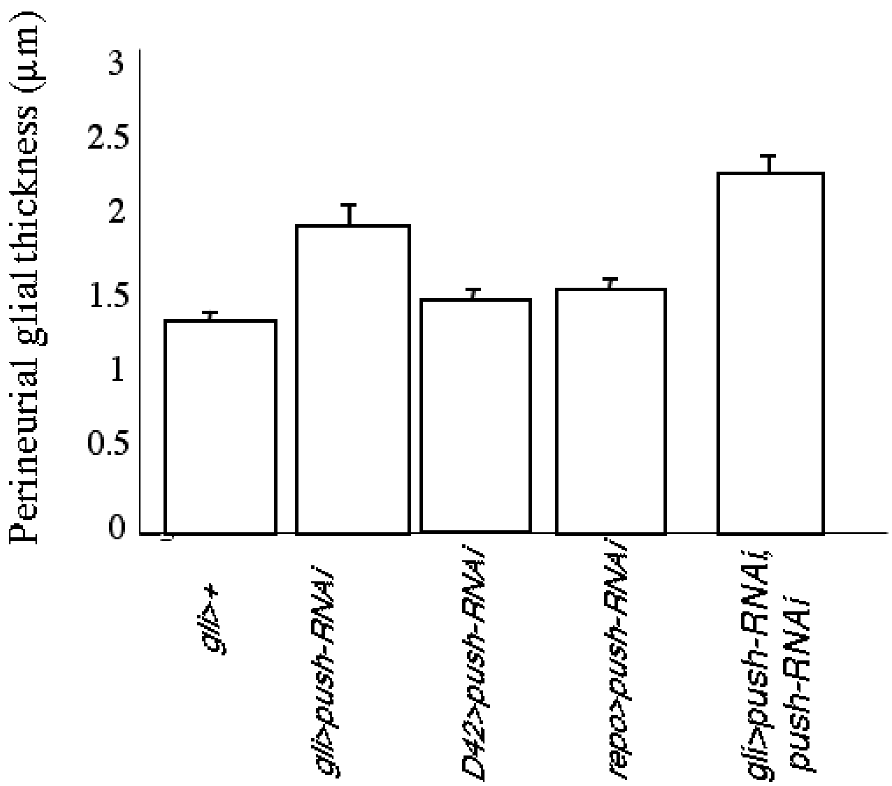


Figure 2: Effects of *push* knockdown in neurons or glia on perineurial glial thickness. Means and SEMs of perineurial glial thickness (Y axis) for the indicated genotypes (X-axis). Number of nerves measured: *gli>+*, n=13, *gli>push-RNAi*, n=19, *D42>push-RNAi*, n=37, *repo>push-RNAi*, n=33, *repo>push-RNAi, push-RNAi*, n=26. *gli>+* is significantly different from *gli>push*$^{RNAi}$*, push*$^{RNAi}$ (p=0.0090).

*Second, to identify the cell(s) in which Push is present:* We decided to address this question by constructing a *push* transgene containing about 10-15 kb of upstream sequence, to enable proper tissue-and temporal-specific expression, *push* genomic sequence (about 18 kb, including about 16 kb of open reading frame) with the C terminus "tap-tagged", which will enable a number of protein chemistry experiments to be performed on Push, including Push localization through immunocytochemistry. Because the DNA is so large in amount, we needed to use a novel in vivo recombination technique to make the construct; this technique is called " recombineering". Student Curtis Lin has successfully completed the construct and has sent the DNA to a company that will inject the DNA into fly embryos so that we can obtain transgenic flies. Because standard P element mediated transformation does not work effectively with DNA of such large size, we used the new phi31 recombinase system to obtain transgenics. Unfortunately, despite screening the progeny of several hundred injected larvae, Curtis was unable to obtain a single recombinant.

Task Three (completed): The major focus of this task was to identify signalling pathways downstream of *NF1* and *Ras* acting in the peripheral glia. Unexpectedly, we found that the PI3K pathway, but not the Raf pathway, appeared to be the most important for mediating the effects of *NF1* and *Ras* on perineurial glial growth. We further showed that PI3K exerted its effects on growth via the downstream kinase Akt and the transcription factor Foxo. These experiments were published last year (Lavery et al., 2007) and the PDF for this paper is appended to this document. The experiments described in this paper are summarized below. Figures from the attached paper are referenced when appropriate.

We also modified the statement of work to begin testing the possibility that the Drosophila metabotropic glutamate receptor, DmGluRA, is an endogenous activator of PI3K in neurons. Given the critical importance of PI3K in mediating the effects of Neurofibromin on glial growth, the possibility that mGluRA endogenously regualates PI3K in neurons (and perhaps glia as well) potentially has huge significance for the mechanisms by which Neurofibromin affects neuronal and glial properties. In fact, the data collected in this task were used as preliminary data for a new award from the CDMRP NFRP to test the possibility that glutamate activation of DmGluRA activates PI3K in glia as well. Our demonstration that ligand activation of DmGluRA in larval motor nerve terminals activates PI3K, and that this PI3K activation increases neuronal growth and arborization via the Tor/S6 Kinase pathway and decrease s neuronal excitability via inhibition of the transcription factor Foxo, was published recently in PLoS Genetics (Howlett et al., 2008). This paper was also listed on the CDMRP website as a Neurofibromatosis Research Highlight in February, 2009 (URL: http://cdmrp.army.mil/highlights/default.htm#4).

To evaluate the role of Ras signalling in nonautonomous growth control within peripheral nerves, we used the *GAL4/UAS* system to express wildtype and mutant transgenes specifically within the peripheral glia. Two *GAL4* drivers, *gli-GAL4* and *MZ709*, were reported to express in the peripheral glia but not the neurons of peripheral nerves. The *gli-Gal4* driver is a particularly well characterized marker for peripheral glia. *gli-Gal4* was generated via gene conversion from a *gli-lacZ* enhancer trap line, which was reported to express specifically in peripheral glia, exit glia and some midline glia. The *gli-Gal4* driver was used to study peripheral glial dynamics during embryonic peripheral nerve development. This driver was also used to study peripheral glial anatomy during larval growth and at the mature third instar larval neuromuscular junction and peripheral sensory structures. These studies confirmed that *gli-Gal4* is expressed in peripheral glia but not motor and sensory neurons.

To confirm that *gli-Gal4* and *MZ709* do not express *Gal4* in the perineurial glia, we visualized the expression pattern of these drivers within peripheral nerves via induced expression of a nuclear-localized GFP. We also visualized the total complement of peripheral nerve nuclei (peripheral and perineurial glial) via the Hoechst DNA dye. As shown in Figures 1B and 1D (from Lavery et al., 2007, attached), there are about 20 nuclei per mm of peripheral nerve. Most of these are perineurial glial nuclei whereas a few are peripheral glial nuclei. If *gli-Gal4* and *MZ709* express in the perineurial glia as well as peripheral glia, then we anticipate that in *gli>GFP(nls)*and *MZ709>GFP(nls),* most or all of these nuclei would contain GFP. In fact, as shown in Figure 1C and 1E (from Lavery et al., 2007, attached), we observe that only a few (presumably peripheral glial) nuclei from these larvae express GFP. Therefore, we conclude that neither *gli-GAL4* and *MZ709* expresses *Gal4* in the perineurial glia. We generally observe GFP in fewer than 8 peripheral glial nuclei, which presumably results from cell-to-cell variability in *Gal4* expression levels, as was reported previously for peripheral glia . We also observed that each driver also expresses *Gal4* within certain cells of the ventral ganglion, as shown in Figure 1 (from Lavery et al., 2007, attached)).

In both mice and humans, neurofibroma formation appears to occur only when the Schwann cell component of the peripheral nerve is homozygous for *$Nf1^-$*. This observation suggests that activated Ras within Schwann cells is necessary for neurofibroma formation. To test the effects of activating Ras within Drosophila peripheral glia (analogous to the mammalian Schwann cell), we used the *gli-GAL4* driver to express $Ras^+$ or the constitutively-active $Ras^{V12}$ specifically in the peripheral glia. We found that larvae bearing *gli-GAL4* and either of two *UAS-*$Ras^{V12}$ transgenes exhibited a thickened perineurial glia. The thickness observed, 2.1-2.3 µm, was significantly (about 50%) greater than the value observed in larvae carrying *gli-GAL4* or *UAS-*$Ras^{V12}$ alone, or *gli>*$Ras^+$ as shown in Figure 2 (from Lavery et al., 2007, attached). We conclude that Ras activation specifically within the peripheral glia is sufficient to promote perineurial glial growth. We also found that *gli-GAL4*-driven co-expression of both *UAS-*$Ras^{V12}$ transgenes does not cause a further increase in perineurial glial thickness: perineurial glial thickness in larvae expressing both transgenes is the same as in larvae expressing either transgene alone, as shown in Figure 2 (from Lavery et al., 2007, attached). This observation suggests that in *gli>*$Ras^{V12}$ larvae, $Ras^{V12}$ levels are not limiting for promoting perineurial glial growth. To rule out the possibility that the presence of two transgenes decreased expression of both via titration of Gal4, we measured perineurial glial thickness in larvae co-expressing *$Ras^{V12}$* with an indifferent transgene (*GFP*). We found that this co-expression did not suppress the growth-promoting effects of *$Ras^{V12}$* , as shown in Figure 2 (from Lavery et al., 2007, attached), suggesting that the presence of a second *UAS*-driven transgene does not significantly affect expression of the first.

Activated Ras activates a number of downstream molecules, including Raf, PI3 kinase (PI3K) and the guanine nucleotide exchange factor for the Ral GTPase. To identify the effector(s) responsible for transducing the nonautonomous growth activation conferred by $Ras^{V12}$, we expressed transgenes encoding the constitutively active $Raf^{F179}$, PI3K-CAAX, and $Ral^{V20}$ proteins within peripheral glia using *gli-GAL4.* As shown in Figure 3B (from Lavery et al., 2007, attached), we found that expression of *$Raf^{F179}$* or *$Ral^{V20}$* had no significant effect on perineurial glial thickness. However, expression of *PI3K-CAAX* increased perineurial glial thickness to about 3 µm as shown in Figure 3A and 3B (from Lavery et al., 2007, attached). This thickness is significantly greater than both wildtype thickness and the increased thickness conferred by *$Ras^{V12}$* expression. When *UAS-PI3K-CAAX* was expressed with a second peripheral glial driver, *MZ709*, perineurial glial thickness was increased to the same extent as with *gli-GAL4*. These results suggest that Ras exerts its nonautonomous effects on perineurial glial growth via activation of PI3K. The observation that *PI3K-CAAX* exerts a stronger effect than *$Ras^{V12}$* might indicate that PI3K levels are limiting in peripheral glia to promote perineurial glial growth. In this view, transgene-induced overexpression of *PI3K-CAAX* overcomes this limitation and enables a more robust growth effect to be observed.

The results shown in Figure 3 (from Lavery et al., 2007, attached) demonstrate that PI3K activation in peripheral glia is sufficient to increase perineurial glial growth. To determine if PI3K activity is necessary for the nonautonomous growth-promoting effects of $Ras^{V12}$, we introduced the heteroallelic *PI3K* loss of function combination *$PI3K^{2H1}/PI3K^{A}$* into *gli>$Ras^{V12}$* larvae. This mutant combination was chosen because it decreases PI3K activity sufficiently to confer phenotypes, but retains sufficient activity to permit viability to the third instar larval stage. We found that *$PI3K^{2H1}/PI3K^{A}$* significantly suppressed the growth-promoting effects of *$Ras^{V12}$* as shown in Figure 4 (from Lavery et al., 2007, attached), which demonstrates that PI3K activity is necessary for this effect. To determine if PI3K activity is necessary in peripheral glia, rather than the perineurial glia, we blocked PI3K activity specifically in the peripheral glia by co-expressing *$Ras^{V12}$* with a transgene encoding the dominant-negative $PI3K^{D954A}$. We found that the peripheral-glial-specific expression of *$PI3K^{D954A}$* blocked the growth-promoting effects of *$Ras^{V12}$* as shown in Figure 4 (from Lavery et al., 2007, attached), suggesting that PI3K activity is required in the peripheral glia to promote perineurial glial growth. In contrast, as described above, co-expressing *$Ras^{V12}$* with *GFP* did not suppress the growth-promoting effects of *$Ras^{V12}$* (see Figure 2).

To confirm that *$PI3K^{2H1}/PI3K^{A}$* suppressed the $Ras^{V12}$ phenotype by decreasing PI3K activity in the peripheral glia rather than the perineurial glia, we introduced *$PI3K^{2H1}/PI3K^{A}$* into *gli>PI3K-CAAX* larvae. The extremely thick perineurial glia conferred by *PI3K-CAAX* was not significantly affected by the presence of *$PI3K^{2H1}/PI3K^{A}$* as shown in Figure 4 (from Lavery et al., 2007, attached); thus, the perineurial glia in the *$PI3K^{2H1}/PI3K^{A}$* mutant is fully competent to respond to growth promoting signals from the peripheral glia, which strongly suggests that the significant suppression of the *$Ras^{V12}$* growth phenotype by *$PI3K^{2H1}/PI3K^{A}$* results from loss of PI3K activity in the peripheral glia.

One PI3K effector is the protein kinase Akt. Elevated PI3K activity promotes the ability of the kinase PDK1 to phosphorylate and activate Akt. To determine if Akt activity was necessary for the growth-promoting effects of PI3K, we replaced either one or both copies of *$Akt^{+}$* with the strong hypomorphic *$Akt^{4226}$* allele in *gli>PI3K-CAAX* larvae. We found that replacing one copy of *$Akt^{+}$* moderately suppressed, and replacing both copies of *$Akt^{+}$* profoundly suppressed, the effects of *PI3K-CAAX* as shown in Figure 5 (from Lavery et al., 2007, attached). These results demonstrate that Akt activity is required for the growth-promoting effects of PI3K. Akt activity can promote growth cell autonomously. Thus *$Akt^{4226}$* could suppress the growth-promoting effects of PI3K-CAAX by decreasing Akt activity in either the peripheral or perineurial glia. To determine if *$Akt^{+}$* activity in the peripheral glia was sufficient to increase perineurial glial growth, we measured perineurial glial thickness in larvae expressing either of two *UAS-$Akt^{+}$* transgenes driven by *gli-Gal4* and found no effect on the perineurial glia as shown in Figure 5 (from Lavery et al., 2007, attached). Because these *$Akt^{+}$* transgenes encode wildtype Akt, which requires activation by the PI3K-dependent kinase PDK1, it was possible that this lack of effect might result from low endogenous PI3K activity in the peripheral glia, which would lead to inability to activate Akt. To test this possibility, we activated Akt in the peripheral glia by using *gli-Gal4* to co-overexpress *UAS-$Akt^{+}$* with *UAS-PI3K-CAAX*. We found a striking increase in perineurial glial thickness in this genotype compared with larvae overexpressing *PI3K-CAAX* alone as shown in Figure 5 (from Lavery et al., 2007, attached); note that the *gli>PI3K-CAAX, $Akt^{+}$* nerve pictured is an extreme nerve, not a typical nerve. This result suggests that in the presence of activated PI3K, Akt levels within the peripheral glia become limiting for activating growth nonautonomously. In this view, increasing Akt levels by transgene expression serves to relieve this limitation and enable further increase in perineurial glial growth. We conclude that Akt activation in the peripheral glia is sufficient to increase perineurial glial growth.

In addition to the effect of *gli>PI3K-CAAX, Akt* on perineurial glial thickness, we observed a significant increase in thickness of the "axon bundle" (motor and sensory axons and peripheral glia) in this genotype. This increased thickness is due mostly to the presence of motor and sensory axons of increased diameter as shown in Figure 5 (from Lavery et al., 2007, attached). A more complete description of this phenotype will be presented in a future study. However, these observations suggest that extremely high levels of Akt activity can nonautonomously activate axonal growth as well as perineurial glial growth.

One Akt effector is the forkhead-box transcription factor FOXO. FOXO inhibits PI3K- and Akt-dependent gene expression; this activity is lost upon phosphorylation by Akt, which causes phospho-FOXO to be excluded from the nucleus. To test the possibility that PI3K and Akt activity increase perineurial glial growth by inhibiting FOXO, we co-expressed *PI3K-CAAX* and either of two *FOXO* transgenes within the peripheral glia. We found that expression of either *FOXO* transgene significantly suppressed the growth-promoting effects of *PI3K-CAAX* as shown in Figure 6 (from Lavery et al., 2007, attached). In contrast, when we co-expressed *PI3K-CAAX* with a neutral *UAS*-driven transgene (*UAS-GFP*), we did not observe significant suppression as shown in Figure 6 (from Lavery et al., 2007, attached). Thus, *FOXO* overexpression suppresses the growth-promoting effects of PI3K.

Our studies provide new mechanistic insights into the nonautonomous growth promoting effects of peripheral glia (Schwann cells) in peripheral nerves. Our results are completely consistent with the possibility that these nonautonomous effects are mediated by a pathway in which the negative regulation of growth by FOXO is inhibited by its Akt-dependent phosphorylation. FOXO might directly or indirectly repress transcription of growth factors that recruit the growth of neighbors

We have also begun attempts to identify growth factors that may be responsible for the nonautonomous growth promoting effects of PI3K on perineurial glial growth. Given that Foxo is a transcription factor, we hypothesized that Foxo regulates perineurial glial thickness nonautonomously by repressing the transcription of a growth factor released from the peripheral glia that activated growth of the perineurial glia. This Foxo-mediated repression could be indirect. If so, then overexpressing this growth factor with the *Gal4/UAS* system would be predicted to increase perineurial glial growth. With this hypothesis in mind, we overexpressed several candidate growth factor genes in peripheral glia and measured perineurial glial thickness. Because the Ratner lab had previously implicated aberrant EGF signalling in neurofibroma formation, we first tested EGF ligands, and TGF-β. Because the Mirsky lab had implicated Desert Hedge Hog in perineurial glial development, we also tested the role of Hh ligands in perineurial glial growth control. However, we found that overexpression of none of these ligands affected perineurial glial thickness (Figure 3). Because it has been reported that growth factors or peptide hormones are more effective when overexpressed in the recipient cell, rather than the signalling cell, we also overexpressed many of these ligands in the recipient cell (perineurial glia) using the *repo-Gal4* driver. We found a moderate effect of *spitz* overexpression in perineurial glia (Figure 3). This possible role of the *spitz* EGF ligand will be investigated further by expressing *spitz* RNAi in peripheral glia, and seeing if this expression suppresses the increased perineurial glial growth conferred by PI3K overexpression.

We have also investigated the roles of the Ras-activated Raf and Ral transgenes as collaborators with PI3K in activating perineurial glial cell growth. Our evidence that the Ral GTPase might be involved in nonautonomous growth regulation came from experiments performed for other reasons. As reported previously we had demonstrated that activating Ras in peripheral glia promotes perineurial glial growth (Lavery et al., 2007), but we then wanted to know if Ras activity might also be required in the perineurial glia to respond properly to the growth promoting effects of the peripheral glia. To test this possibility, we introduced a heteroallelic loss of function combination of *Ras* ($Ras^{e2F}/Ras^{12A}$) into larvae expressing activated PI3K in the peripheral glia. We found that the *Ras* alleles significantly suppressed the growth promoting effect of activated PI3K (Figure 4 below). To show that Ras was exerting this effect in the perineurial glia, rather than the peripheral glia, we wanted to show that blocking Ras specifically in the peripheral glia (by expressing the dominant-negative $Ras^{N17}$ transgene) was not able to suppress the growth-promoting effects of activated PI3K. But to our great surprise, we found that co-expression of $Ras^{N17}$ completely suppressed the growth-promoting effects of PI3K (Figure 3 below). In contrast, co-expressing the constititutively-active $Ras^{V12}$ enhanced the growth-promoting of PI3K (Figure 4 below).

Because PI3K-CAAX, the constitutively active allele that we used to promote growth is predicted to be Ras-independent, we thought it unlikely that $Ras^{N17}$was decreasing PI3K activity. Rather, we thought it was more likely that $Ras^{N17}$ was blocking a second Ras-activated signalling pathway that was required for PI3K to induce growth. This second pathway did not appear to be the Raf-Erk pathway because co-expression of either a dominant-negative or constitutively-active *Raf* transgene with *PI3K-CAAX* had only moderate effects on perineurial glial thickness (Figure 4 below). The Ral-GTPase represents a third, Ras-activated pathway: active Ras activates Ral via the Ral guanine dissociation factor.

To test the possibility that Ral is involved in the control of perineurial glial growth, we expressed the constitutively active *Ral*$^{V20}$ transgene in peripheral glia and found no effect on perineurial glial growth (Figure 4 below). However, expression of *Ral*$^{V20}$ significantly enhanced the ability of PI3K-CAAX to promote perineurial glial growth (Figure 5 below), and this enhancement was approximately the same as the enhancement conferred by co-expression with *Ras*$^{V12}$. These results support the possibility that Ral activity regulates the ability of PI3K to promote peripheral nerve growth nonautonomously.

The experiments described above suggest that Ral is one Ras effector with activity required to potentiate the effects of PI3K. To determine if Ral is the only Ras effector with activity required to potentiate the effects of PI3K, we decided to test if expression of activated Ral was sufficient to enhance the effects of PI3K-CAAX even in the simultaneous presence of Ras$^{N17}$. If so, then such a result would indicate that PI3K and Ral could together promote perineurial glial growth even when every other Ras effector pathway was blocked by Ras$^{N17}$. We first tested the system with a positive control. We coexpressed *PI3K-CAAX* and *Ras*$^{N17}$, in which perineurial glial growth is suppressed, along with *Ras*$^{V12}$, which is anticipated to be completely epistatic to *PI3K-CAAX* and *Ras*$^{N17}$ because Ras$^{N17}$ blocks wildtype Ras by blocking the nucleotide exchange factor, which Ras$^{V12}$ does not require for activity.

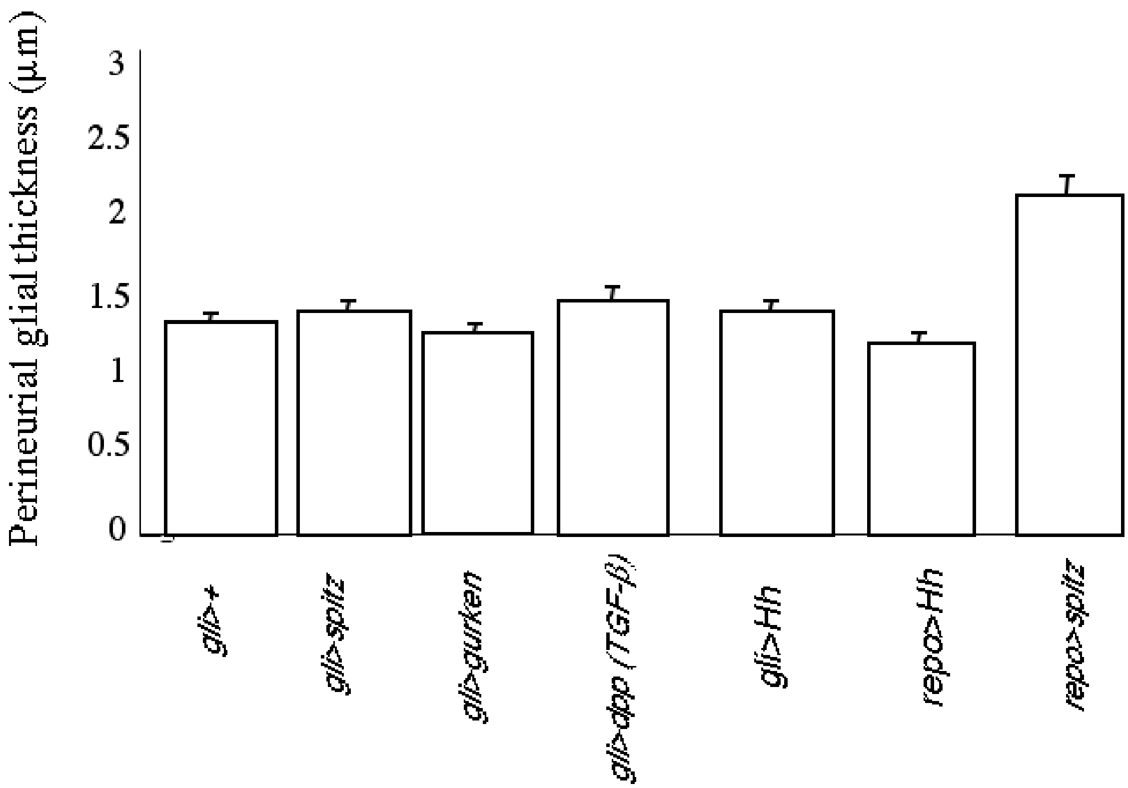


Figure 3: Effects of growth factor misexpression on perineurial glial growth. Means and SEMs of perineurial glial thickness (Y axis) for the indicated genotypes (X-axis). Number of nerves measured: *gli*>+, n=13, *gli>spitz,* n=33, *gli>gurken*, n=34, *gli>dpp*, n=20, *gli>Hh,* n=21, *repo>Hh,* n=28, *repo>spitz,* n=32. No pairwise combinations have statistically significant differences.

Figure 4:

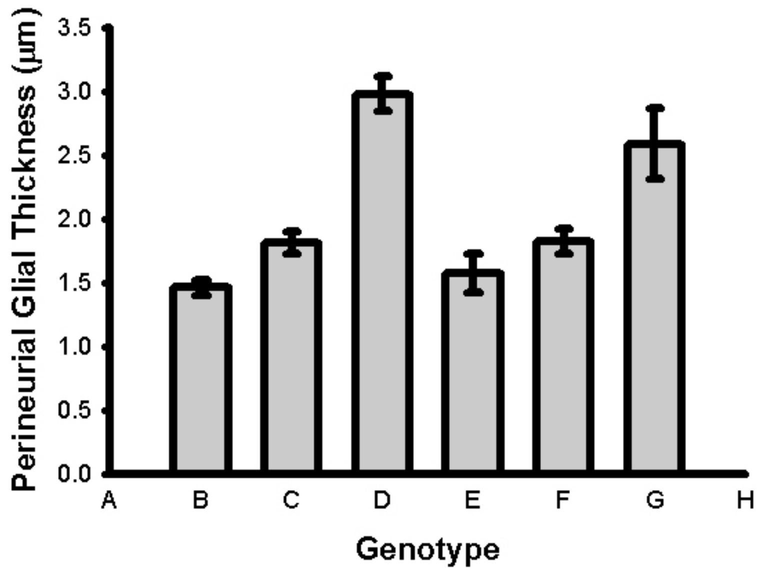


Figure 4: Ras activity in the peripheral glia is required for PI3K to increase perineurial glial growth. Y Axis: Means +/- SEMS of perineurial glial thickness (µm) for the indicated genotypes (X Axis). Genotypes were as follows: A: *gli-Gal4/+*, n=60, B: *UAS-PI3K-CAAX/+*, n=59, C: *gli>PI3K-CAAX*, n=76, D: *gli>PI3K-CAAX; Ras*$^{12A}$*/Ras*$^{e2F}$, n=30, E: *gli>PI3K-CAAX, Ras*$^{N17}$, n=31, F: *gli>PI3K-CAAX, GFP*, n=25.

Figure 5:

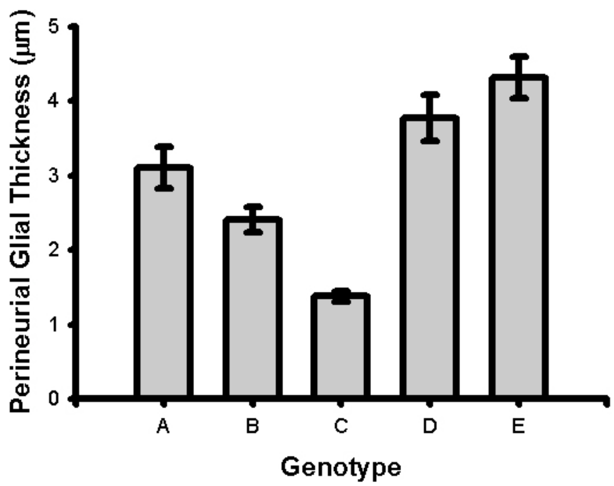


Figure 5: Ral, but not Raf potentiates the effects of PI3K on perineurial glial growth. Y Axis: Means +/- SEMS of perineurial glial thickness (µm) for the indicated genotypes (X Axis). Genotypes were as follows: A: *gli>PI3K-CAAX, Raf*$^{gof}$, n=52, B: *g.i>PI3K-CAAX, Raf*$^{DN}$, n=24, C: *gli>Ral*$^{V20}$, n=29, D: *gli>PI3K-CAAX, Ras*$^{V12}$, n=39, and E: *gli>PI3K-CAAX, Ral*$^{V20}$, n=30.

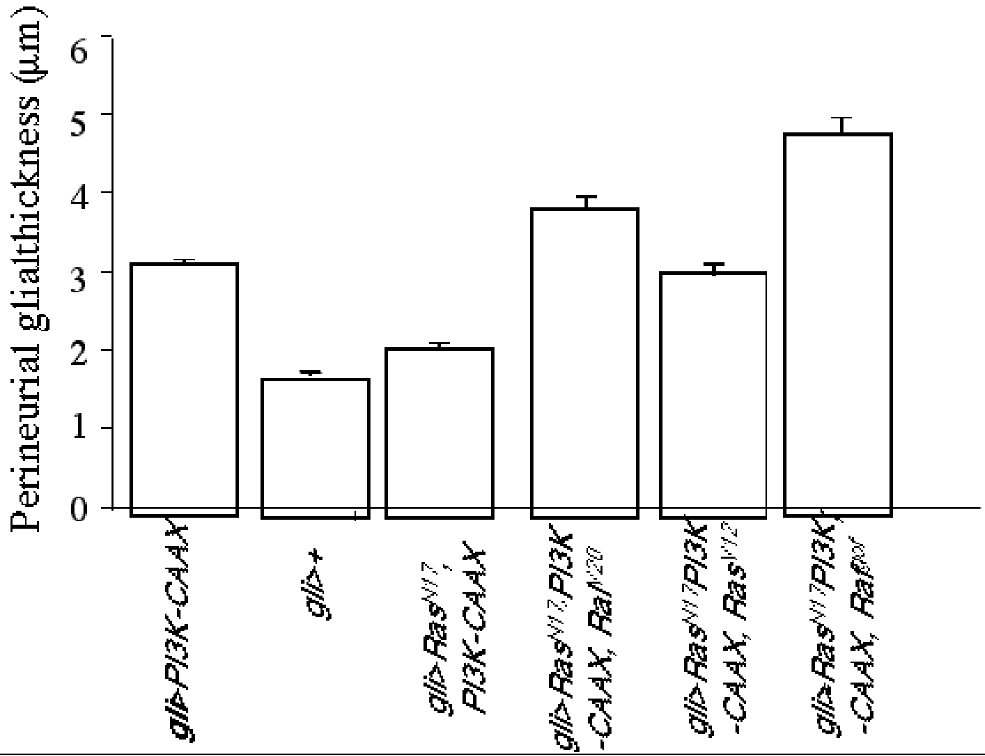


Figure 6: Interactions between PI3K and Ras, Raf and Ral. Means and SEMs of perineurial glial thickness (Y axis) for the indicated genotypes (X-axis). Number of nerves measured: *gli>PI3K-CAAX,* n=76, *gli>+*, n=13, *gli>Ras$^{N17}$, PI3K-CAAX*, n=31, *gli>Ras$^{N17}$, PI3K-CAAX, Ral$^{V20}$*, n=22, *gli>Ras$^{N17}$, PI3K-CAAX, Ras$^{V12}$*, n=26, *gli>Ras$^{N17}$, PI3K-CAAX, Raf$^{gof}$*, n=20. The following pairwise combinations have statistically significant differences versus *gli>PI3K-CAAX*: *gli>+* ($p<0.0001$), *gli>PI3K-CAAX, Ras$^{N17}$* ($p<0.0001$), *gli>PI3K-CAAX, Ras$^{N17}$, Ral$^{V20}$* ($p=0.0015$), *gli>PI3K-CAAX, Ras$^{N17}$, Raf$^{gof}$* ($p<0.0001$).

As expected, we found that increased perineurial glial growth was restored when *PI3K-CAAX*, *Ras$^{N17}$* and *Ras$^{V12}$* were each expressed in the peripheral glia (Figure 6). In addition, we found out that, as expected, increased perineurial glial growth was restored when *PI3K-CAAX*, *Ras$^{N17}$* and *Ral$^{V20}$* were each expressed in the peripheral glia (Figure 6). However, when we co-expressed *PI3K-CAAX*, *Ras$^{N17}$* and *Raf$^{gof}$* in the peripheral glia, increased perineurial glial growth was still restored (Figure 6), even though Ral activity is predicted to be blocked in this genotype. This result was both unexpected and difficult to interpret, particularly as perineurial glial growth was much thicker in larvae expressing *PI3K-CAAX*, *Ras$^{N17}$* and *Raf$^{gof}$* than in larvae only expressing *PI3K-CAAX* and *Raf$^{gof}$*. Taken at face value, this result suggests that *Ras$^{N17}$* has a positive effect on perineurial glial growth, at least under some conditions. To my knowledge, there is no precedent in the literature that would be consistent with this possibility. At this point, I think it is best to drop this aspect of the project until some way of moving forward comes to light (for example, by a new publication that suggests a mechanism for this observation).

Finally, we evaluated the role of the PI3K-regulated Tor pathway in the nonautonomous control of perineurial glial growth. As described last year, we used transgenes encoded altered S6 Kinase (S6K), which is phosphorylated and activated by Tor, as our way to manipulate Tor activity. We found that expressing a constitutively-active S6K *S6K$^{act}$* specifically in the peripheral glia was not sufficient to increase perineurial glial growth (Figure 7). Furthermore, elimination of Foxo by the *Foxo$^{21}$/Foxo$^{25}$* heteroallelic null combination also failed to increase perineurial glial growth (Figure 7). To determine if S6K activity is necessary to mediate the effects of PI3K-CAAX on perineurial glial growth, we co-expressed *PI3K-CAAX* and the dominant-negative *S6K$^{DN}$* in peripheral glia, and we found that the presence of *S6K$^{DN}$* significantly suppressed the ability of *PI3K-CAAX* to increase perineurial glial growth (Figure 7). These results suggest that PI3K-mediated activation of the Tor pathway, in addition to inhibition of Foxo, is necessary for full activation of perineurial glial growth. This result raised the possibility that PI3K activated glial growth in two ways: by activating transcription of the EGF-ligand *spitz*, and by activating the Tor pathway. If so, then increased perineurial glial growth would require both overexpression of *spitz* and expression of $S6K^{act}$. To test this possibility, we meausred perineurial glial growth in larvae overexpressing both $S6K^{act}$ and *spitz* in

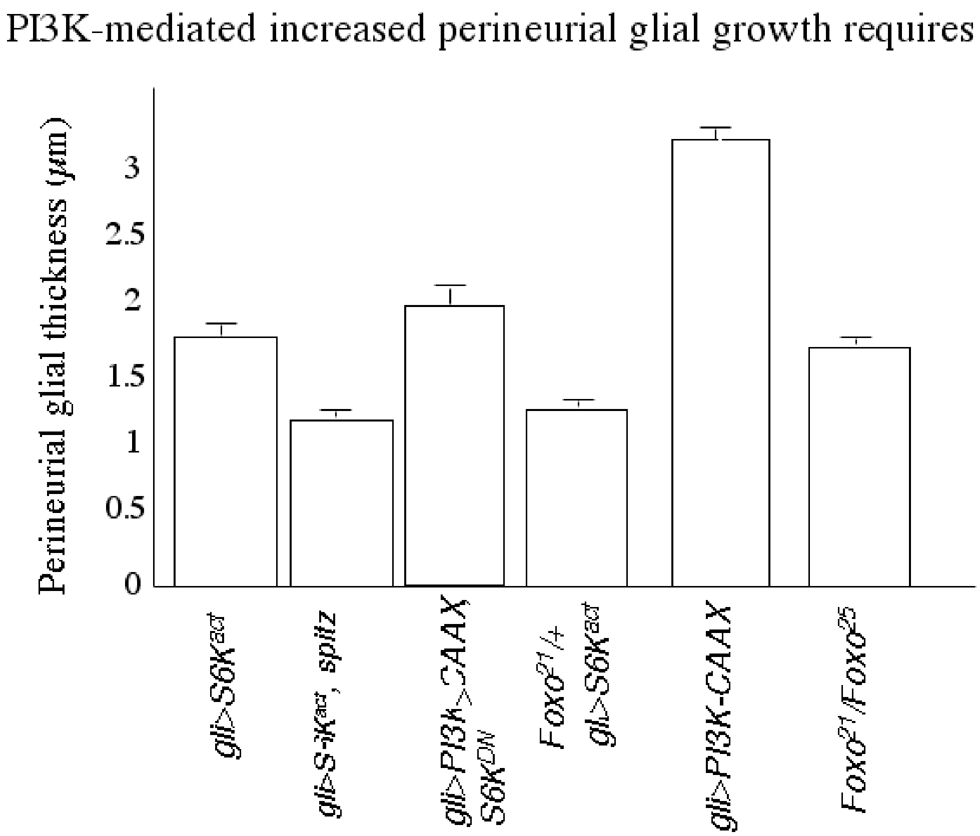


Figure 7: Means and SEMs of perineurial glial thickness (Y axis) for the indicated genotypes (X-axis). Number of nerves measured: *gli>PI3K-CAAX,* n=76, *gli>*$S6K^{act}$, n=26, *gli>* $S6K^{act}$, *spitz*, n=13, *gli>*$S6K^{DN}$*, PI3K-CAAX*, n=32, $Foxo^{21}/+$*; gli>*$S6K^{act}$ n=31, $Foxo^{21}/Foxo^{25}$, n=24. The pairwise combination *gli>PI3K-CAAX* vs. *gli>PI3K-CAAX,* $S6K^{DN}$ is significantly different ($p<0.0001$).

peripheral glia, but found no increase in glial thickness (Figure 7). We also found no evidence of increased perineurial glial growth when we combined expression of $S6K^{act}$ and loss of one dose of *Foxo* (Figure 7). Therefore we tentatively conclude that PI3K-mediated increased perineurial growth requires both inhibition of Foxo and activation of the Tor pathway.

Regulation of neuronal PI3K by the Drosophila metabotropic glutamate receptor: This task, following revision of the original task #4, is based on two papers published after this proposal was submitted for funding. The papers are: Bogdanik et al. (2004), and Martin-Pena et al. (2006). Bogdanik et al. reported effects of mutations in the single Drosophila metabotropic glutamate receptor (mGluRA) on motor neuron structure and function, whereas Martin-Pena et al. reported effects of altered PI3K activity on Drosophila motor neuron structure and function. The similarity, at least superficially, in phenotype between these genotypes raised the possibility that activation of the Drosophila mGluRA in motor neurons activates PI3K. Given the importance of PI3K in mediating the effects of Neurofibromin on various aspects of cell biology, the possibility that glutamate-liganded metabotropic glutamate receptors could act as endogenous activators of PI3K would have huge significance in the field.  Therefore we embarked on a series of experiments to test this possibility, and identify downstream signalling pathways mediating the effects of PI3K on neuronal growth and excitability.  As described above, this work was recently published in PLoS Genetics (Howlett et al., 2008).  A PDF of this paper is appended to this report.

The increase in neuronal excitability conferred by the *mGluRA$^{112b}$* null mutation is manifested by an increased rate of onset of a form of synaptic plasticity termed long-term facilitation (LTF), which is induced when a motor neuron is subjected to repetitive nerve stimulation at low bath [$Ca^{2+}$].  At a certain point in the stimulus train, an abrupt increase in transmitter release and hence muscle depolarization (termed excitatory junctional potential, or ejp) is observed (Figure 1 from Howlett et al., attached).  This abrupt increase is caused by an abrupt increase in the duration of nerve terminal depolarization and hence $Ca^{2+}$ influx, and reflects a progressive increase in motor neuron excitability induced by the repetitive nerve stimulation:  when an excitability threshold is reached, LTF occurs.

In Drosophila, many genotypes that increase motor neuron excitability by decreasing $K^+$ currents or increasing $Na^+$ currents increase the rate of onset of LTF.  For example, altered activities of *frequenin* and *Hyperkinetic*, which act via $K^+$ channels, or *paralytic* and *pumilio*, which act via $Na^+$ channels, each increase the rate of onset of LTF.  By increasing motor neuron excitability, these genotypes apparently bring excitability closer to the threshold required to evoke LTF and consequently decrease the number of prior nerve stimulations required to reach this threshold. The observation that *mGluR$^{112b}$* increases the rate of onset of LTF suggested that *mGluRA$^{112b}$* increases motor neuron excitability as well.

The increased excitability of *mGluRA$^{112b}$* led Bogdanik et al. (2004) to suggest that mGluRA mediates an activity-dependent inhibition of neuronal excitability.  In this view, glutamate release from motor nerve terminals downregulates subsequent neuronal activity by activating presynaptic mGluRA autoreceptors, which then decrease excitability.  Elimination of mGluRA disrupts this negative feedback and prevents the decrease in excitability from occurring.

**The *mGluRA$^{112b}$* null mutation increases neuronal excitability by preventing PI3K activation:** In addition to increasing neuronal excitability, *mGluRA$^{112b}$* also decreased arborization and synapse number at the larval neuromuscular junction.  This phenotype is also observed in larval motor neurons with decreased activity of PI3K. This observation raised the possibility that mGluRA might exert its effects on neuronal excitability as well as synapse formation via PI3K activity.  To test the possibility that PI3K mediates the effects of mGluRA on neuronal excitability, we used the *D42 Gal4* driver to overexpress transgenes expected to alter activity of the motor neuron PI3K pathway.  We found that inhibiting the PI3K pathway by motor neuron-specific overexpression of either the phosphatase *PTEN*, which opposes the effect of PI3K, or the dominant-negative *PI3K$^{DN}$*, each significantly increased the rate of onset of LTF, similarly to that of *mGluRA$^{112b}$* (Figure 1, Howlett et al., attached).  In contrast, we found that activating the PI3K pathway by expression of the constitutively active *PI3K-CAAX*, or via RNAi-mediated inhibition of *PTEN*, decreased rate of onset of LTF (Figure 1, Howlett et al., attached).

In addition to effects on LTF, mutations that alter motor neuron excitability can alter basal transmitter release and hence ejp amplitude at low bath $Ca^{2+}$ concentrations, at which $Ca^{2+}$ influx would be limiting for vesicle fusion to occur.  For example, mutations in *ether-a go-go (eag)*, which encodes a potassium channel $\alpha$ subunit, increase transmitter release about two-fold, whereas a mutation in the sodium channel gene *paralytic* decreases transmitter release by increasing the frequency at which nerve stimulation failed to evoke any vesicle fusion, termed "failure" of vesicle release.  Presumably altered excitability affects the amplitude or duration of the action potential and consequently the amount of $Ca^{2+}$ influx through voltage-gated channels.  We found that *mGluRA$^{112b}$* also increased ejp amplitude and hence basal transmitter release at three low bath $Ca^{2+}$ concentrations tested (Figure 1C, Howlett et al., attached), which is consistent with increased motor neuron excitability in this genotype.  We found that decreasing PI3K pathway activity via motor neuron overexpression of *PI3K$^{DN}$* or *PTEN* also increased transmitter release to levels similar to *mGluRA$^{112b}$*, whereas increasing PI3K pathway activity via overexpression of *PI3K-CAAX* decreased basal transmitter release (Figure 1C, Howlett et al., attached).

The *mGluRA$^{112b}$* mutation also decreased the frequency at which failures of vesicle release occur, particularly at the lower $Ca^{2+}$ concentrations tested (Figure 1D, Howlett et al., attached).  This observation confirms that the effect of *mGluRA$^{112b}$* on ejp amplitude is presynaptic.  We also observed a decreased frequency of failures when the PI3K pathway was inhibited by motor neuron expression of *PI3K$^{DN}$* or *PTEN* (Figure 1D, Howlett et al., attached).  In contrast, motor neuron overexpression of *PI3K-CAAX* increased the frequency of failures (Figure 1D, Howlett et al., attached). Therefore, with three electrophysiological readouts, the *mGluRA$^{112b}$* mutant phenotype was mimicked by decreased activity of the PI3K pathway, whereas increasing PI3K pathway activity conferred opposite effects.

These observations support the notion that loss of mGluRA increases motor neuron excitability by preventing the activation of PI3K.  If so, then motor neuron expression of *PI3K-CAAX* is predicted to suppress the *mGluRA$^{112b}$* hyperexcitability.  To test this possibility, we drove motor-neuron expression of *PI3K-CAAX* in an *mGluRA$^{112b}$* background and found a rate of onset of LTF, ejp amplitude, and failure frequency very similar to what was observed when *PI3K-CAAX* was expressed in a wildtype background (Figure 1, Howlett et al., attached). We conclude that hyperexcitability of the *mGluRA*$^{112b}$ mutant results from inability to activate PI3K.

**Glutamate application increases levels of phosphorylated Akt in motor nerve terminals in an mGluRA-dependent fashion:** The results described above suggest that glutamate release from motor nerve terminals as a consequence of motor neuron activity activates PI3K within motor nerve terminals via mGluRA autoreceptors. To test this possibility directly, we measured the ability of glutamate applied to the neuromuscular junction to activate PI3K within motor nerve terminals. To assay for PI3K activity we applied an antibody specific for the phosphorylated form of the kinase Akt (p-Akt), which is increased by elevated PI3K pathway activity. The ability to detect p-Akt in larval motor nerve terminals overexpressing *PI3K-CAAX*, but not in wildtype (Figure 2, Howlett et al., attached), validates this antibody as a PI3K reporter.

We compared p-Akt levels in wildtype versus *mGluRA*$^{112b}$ motor nerve terminals immediately prior to or following a 1 minute application of 100 µM glutamate. We found that glutamate application strongly increased p-Akt levels in wildtype larvae, but not in the *mGluRA*$^{112b}$ larvae (Figure 2, Howlett et al., attached), demonstrating that glutamate application increases nerve terminal p-Akt levels, and that mGluRA activity is required for this increase. Furthermore, we found that mGluRA activity was required presynaptically for this p-Akt increase: motor neuron-specific expression of an *mGluRA* RNAi construct, which was previously shown to decrease mGluRA levels successfully, blocked the ability of glutamate to increase p-Akt levels, as did motor motor neuron-specific expression of *PI3K*$^{DN}$ (Figure 2, Howlett et al., attached). Thus, presynaptic mGluRA and PI3K activity are both necessary for glutamate to increase p-Akt.

**The effects of PI3K on neuronal excitability are mediated by Foxo, not Tor/S6 kinase:** Many effects of the PI3K pathway are mediated by the downstream kinase Akt. Activated Akt phosphorylates targets such as Tsc1/Tsc2, which regulates cell growth via the Tor/S6 Kinase (S6K) pathway, Foxo, which regulates apoptosis, and GSK3, which mediates at least in part the effects of altered PI3K pathway activity on arborization and synapse number. All of these Akt-mediated phosphorylation events inhibit activity of the target protein.

If PI3K pathway activity decreases neuronal excitability by inhibiting Foxo, then Foxo overexpression is predicted to mimic the hyperexcitability observed when PI3K pathway activity is blocked in motor neurons, whereas loss of Foxo is predicted to mimic the hypoexcitability observed when *PI3K-CAAX* is expressed in motor neurons. To test these predictions, we measured the rate of onset of LTF in larvae carrying the heteroallelic *Foxo*$^{21}$/*Foxo*$^{25}$ null mutant combination(Junger et al. 2003) and in larvae overexpressing *Foxo*$^{+}$ in motor neurons. We found that overexpression of *Foxo*$^{+}$ increased the rate of onset of LTF to a level very similar to that observed when PI3K pathway activity was decreased (Figure 3A, Howlett et al., attached), whereas in *Foxo*$^{21}$/*Foxo*$^{25}$ larvae, the rate of onset of LTF, basal transmitter release and frequency of successful ejps were decreased to levels very similar to those observed when *PI3K-CAAX* was expressed in motor neurons (Figure 3, Howlett et al., attached). These observations support the notion that PI3K activity decreases excitability by downregulating Foxo activity.

If the hyperexcitability conferred by motor neuron expression of *PI3K*$^{DN}$ results from Foxo hyperactivity, then the *Foxo*$^{21}$/*Foxo*$^{25}$ null combination will suppress this hyperexcitability and confer motor neuron hypoexcitability similar to what is observed in *Foxo*$^{21}$/*Foxo*$^{25}$ larvae in an otherwise wildtype background. We confirmed this prediction: larvae carrying the *Foxo*$^{21}$/*Foxo*$^{25}$ null combination and expressing *PI3K*$^{DN}$ in motor neurons exhibited a rate of onset of LTF, basal transmitter release, and failure frequency very similar to what was observed in the *Foxo*$^{21}$/*Foxo*$^{25}$ null mutant alone (Figure 3, Howlett et al., attached), or in larvae expressing *PI3K-CAAX* in motor neurons. We used the *OK6* motor neuron *Gal4* driver rather than *D42* for ease of stock construction in experiments involving *Foxo*$^{21}$/*Foxo*$^{25}$. *OK6* confers motor neuron phenotypes indistinguishable from *D42* in our assays (Figure 3A, Howlett et al., attached and not shown).

In addition, if the hypoexcitability conferred by motor neuron expression of *PI3K-CAAX* results from decreased Foxo activity, then co-overexpression of *Foxo*$^{+}$ will suppress this hypoexcitability and confer hyperexcitability similar to what is observed when *PI3K*$^{DN}$, *PTEN* or *Foxo*$^{+}$ alone are expressed in motor neurons. We confirmed this prediction: larvae co-expressing *Foxo*$^{+}$ and *PI3K-CAAX* in motor neurons exhibited rate of onset of LTF, basal transmitter release and failure frequency very similar to what was observed when *PI3K*$^{DN}$, *PTEN,* or *Foxo*$^{+}$ alone were expressed in motor neurons (Figure 3, Howlett et al., attached). Thus, eliminating *Foxo* reverses the hyperexcitability conferred by blocking PI3K pathway in motor neurons, whereas overexpressing *Foxo*$^{+}$ reverses the hypoexcitability conferred by activating PI3K in motor neurons. These epistasis tests support the notion that PI3K activity decreases motor neuron excitability by inhibiting Foxo.

In contrast, we found that altering the Tor/S6K pathway had no effect on motor neuron excitability. In particular, motor neuron expression of neither the dominant-negative *S6K*$^{DN}$ nor the constitutively active *S6K*$^{Act}$ transgene had any effect on the rate of onset of LTF (Figure 4, Howlett et al., attached). In addition, expression of *S6K*$^{DN}$ had no effect on the ability of *PI3K-CAAX* to decrease the rate of onset of LTF (Figure 4, Howlett et al., attached). Furthermore, expression of *S6K*$^{DN}$ had no effect on basal transmitter release, and did not affect the ability of *PI3K-CAAX* to depress basal transmitter release (data not shown). Therefore we conclude that the Tor/S6K pathway does not mediate the effects of PI3K on neuronal excitability.

**The effects of PI3K on synapse number are mediated by Tor/S6 kinase, not Foxo:** Because altered PI3K pathway activity alters motor neuron arborization and synapse number, it seemed possible that a causal relationship existed between the PI3K-mediated excitability and neuroanatomy defects. To test this possibility, we evaluated the roles of the Tor/S6K and Foxo pathways in mediating the effects of altered PI3K activity on synapse number. We found that motor neuron-specific expression of $S6K^{Act}$ increased synapse number to an extent similar to *PI3K-CAAX,* and motor neuron expression of $S6K^{DN}$ decreased synapse number to the same extent as *PTEN* while also partially suppressing the increase in synapse number conferred by *PI3K-CAAX* (Figure 5B, Howlett et al., attached). These observations suggest that S6K mediates in part the effects of PI3K on arborization and synapse number. However, the ability of $S6K^{DN}$ to suppress only partially the effects of PI3K-CAAX overgrowth suggests that both Tor/S6K and a second, PI3K-mediated, pathway (presumably involving GSK3) regulate synapse formation. In contrast to the effects of altered S6K on synapse formation, we found that $Foxo^{+}$ overexpression had no effect on synapse number (data not shown) and failed to suppress the growth-promoting effects of PI3K-CAAX (Figure 5A and Figure 5B, Howlett et al., attached).

We found that the PI3K pathway also affects growth along the length of axons and thus regulates axon diameter. In Drosophila peripheral nerves, about 80 motor and sensory axons are wrapped by about three layers of glia, as shown in the transmission electron micrograph from cross sections of peripheral nerves in Figure 5C (Howlett et al., attached). We found that motor neuron specific expression of *PTEN* decreased axon diameter, whereas motor-neuron specific expression of *PI3K-CAAX* increased axon diameter. Tor/S6K, but not Foxo, mediates this growth effect. In particular, motor neuron-specific expression of $S6K^{Act}$ increased axon diameter to an extent similar to *PI3K-CAAX*, and motor-neuron-specific expression of $S6K^{DN}$ decreased motor axon diameter to an extent similar to *PTEN* and also partially suppressed the growth-promoting effects conferred by *PI3K-CAAX*. In contrast, $Foxo^{+}$ overexpression had no effect on the ability of PI3K-CAAX to increase axon diameter (Figure 5D, Howlett et al., attached). Therefore, Foxo mediates the excitability effects, but not the growth-promoting effects, of altered PI3K pathway activity, whereas the Tor/S6K pathway mediates in part the growth promoting effects but not the excitability effects of altered PI3K pathway. We conclude that the excitability and growth effects are completely separable genetically and thus have no causal relationship.

**Activity-dependent increase in synapse number requires PI3K activity:** Depending on the system, neuronal activity can either restrict or promote synapse formation). The Drosophila *eag Sh* double mutant, in which two distinct potassium channel subunits are simultaneously disrupted, displays extreme neuronal hyperexcitability, and a consequent increase in synapse number. This activity-dependent increase in synapse number does not require mGluRA activity, suggesting that excessive glutamate release is not necessary for this excessive growth to occur. To determine if PI3K activity is required for this overgrowth, we compared synapse number in wildtype larvae, in larvae expressing dominant-negative transgenes for both *eag* ($eag^{DN}$) and *Sh* ($Sh^{DN}$ in motor neurons, and in larvae co-expressing $eag^{DN}$, $Sh^{DN}$ and $PI3K^{DN}$. We found that co-expression of $eag^{DN}$ and $Sh^{DN}$ in motor neurons increased synapse number similarly to what was observed previously, and that this increase was completely blocked by simultaneous expression of $PI3K^{DN}$ but not by *lacZ* (Figure 6, Howlett et al., attached). Thus, the activity-dependent increase in synapse formation requires PI3K activity. The observation that glutamate activation of mGluRA is not necessary for this increase raises the possibility that another PI3K activator contributes to synapse formation at the larval nmj. Insulin is a plausible candidate for such an activator because both insulin and insulin receptor immunoreactivity are present at the nmj.

**A mechanism for the glutamate-induced negative feedback of motor neuron excitability:** The effects on neuronal excitability of altered mGluRA, PI3K, and Foxo activities are consistent with a model in which glutamate released from motor nerve terminals as a consequence of motor neuron activity activates motor neuron PI3K via mGluRA autoreceptors, which then downregulate neuronal excitability via inhibition of Foxo (Figure 7, Howlett et al., attached). Foxo, in turn, might regulate excitability via transcription of ion channel subunits or regulators.

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KEY RESEARCH ACCOMPLISHMENTS

We found that Ras activity in the peripheral glia nonautonomously promotes perineurial glial growth in Drosophila third instar larvae by activating PI3K and Akt, and consequently inhibiting activity of the transcription factor Foxo.

We found that the Drosophila metabotropic glutamate receptor DmGluRA regulates neuronal growth and excitability by activating PI3K in a glutamate-dependent manner.

We found that the effects of DmGluRA and PI3K on growth and excitability are genetically separable. PI3K regulates growth via the Tor/S6K pathway, whereas PI3K regulates excitability via Foxo.

REPORTABLE OUTCOMES

Presentation to the NNFF Consortium on NF1 and NF2, entitled " Evidence that PI3 Kinase mediates the effects of Ras on perineurial glial growth in Drosophila peripheral nerves" (Aspen, CO, May, 2004).

Presentation to the Drosophila research conference entitled " FOXO mediates the nonautonomous effects of Ras and PI3 Kinase on peripheral nerve growth", by William Lavery and Michael Stern (March, 2006, Houston, TX)

Presentation to the NNFF Consortium on NF1 and NF2 entitled " FOXO mediates the nonautonomous effects of Ras and PI3 Kinase on peripheral nerve growth", by William Lavery, Michael Stern (June, 2006, Aspen, CO).

Presentation to the Drosophila research conference entitled "PI3K regulates neuronal excitability and axonal growth and arborization via distinct effector pathways" by Eric Howlett, William Lavery and Michael Stern (April, 2007, Philadephia, PA).

Presentation to the NNFF Consortium on NF1 and NF2 entitled " Ral, but not Raf, enhances the nonautonomous effects of PI3K on perineurial glial growth in the fly peripheral nerve" by William Lavery and Michael Stern (June, 2007, Park City, UT).

Presentation to the Molecular Neurobiology of Drosophila meeting entitled " PI3K regulates neuronal excitability and axonal growth and arborization via distinct effector pathways", by Eric Howlett, Curtis Lin, William Lavery, Michael Stern. (October, 2007, Cold Spring Harbor, NY)

Manuscript by Lavery, W., Hall, V., Yager, J.C., Rottgers, A., Wells, M.C. and Stern, M. (2007). Phosphatidyl inositol 3-Kinase and Akt nonautonomously promote perineurial glial growth in Drosophila peripheral nerves. *J. Neurosci* **27:** 279-288.

Manuscript by Howlett, E., Lin, C.C., Lavery, W., Stern, M. (2008). A PI3-kinase-mediated negative feedback regulates neuronal excitability. PloS Genetics *4,* e1000277.

CONCLUSIONS

I tentatively conclude that *push* acts in the peripheral glia to control perineurial glial growth. Further experiments will test this conclusion definitively. We have completed and published our analysis demonstrating that Ras activates perineurial glial growth nonautonomously by inhibiting action of the transcription factor FOXO in a PI3K- and Akt-dependent manner. We showed and published that the metabotropic glutamate receptor (DmGluRA) regulates neuronal growth and excitability via PI3K-mediated regulation of Tor/S6K and Foxo, respectively. If DmGluRA similarly regulates PI3K in glia, then this could have important implications for growth control within peripheral nerves.

REFERENCES

Bogdanik, L., Mohrmann, R., Ramaekers, A., Bockaert, J., Grau, Y., et al. (2004). The Drosophila metabotropic glutamate receptor DmGluRA regulates activity-dependent synaptic facilitation and fine synaptic morphology. J. Neurosci *24,* 9105-9116.

Howlett, E., Lin, C.C., Lavery, W., Stern, M. (2008). A PI3-kinase-mediated negative feedback regulates neuronal excitability. PloS Genetics *4,* e1000277.

Junger, M.A., Rintelen, F., Stocker, H., Wasserman, J.D., Vegh, M., et al. (2003) The Drosophila forkhead transcription factor FOXO mediates the reduction in cell number associated with reduced insulin signaling. J Biol *2,* 20.

Lavery, W., Hall, V., Yager, J.C., Rottgers, A., Wells, M.C., Stern, M. (2007). Phosphatidylinositol 3-kinase and Akt nonautonomously promote perineurial glial growth in Drosophila peripheral nerves. J. Neurosci. *27,* 279-288.

Lee, T., Feig, L., Montell, D.J. (1996) Two distinct roles for Ras in a developmentally regulated cell migration. Development *122*, 409-418.

Li, W., Ohlmeyer, J. T., Lane, M. E., and Kalderon, D. (1995). Function of protein kinase A in hedgehog signal transduction and Drosophila imaginal disc development. Cell *80*, 553-562.

Martin-Pena, A., Acebes, A., Rodriguez, J.R., Sorribes, A., de Polavieja, G.G., et al. (2006) Age-independent synaptogenesis by phosphoinositide 3 kinase. J. Neurosci *26,* 10199-10208.

Sepp, K.J., Auld, V.J. (1999) Conversion of lacZ enhancer trap lines to GAL4 lines using targeted transposition in Drosophila melanogaster. Genetics *151*,1093-1101.

The, I., Hannigan, G.E., Cowley, G.S., Reginald, S., Zhong, Y., Gusella, J.F., Hariharan, I.K., Bernards, A. (1997) Rescue of *Drosophila NF1* mutant phenotype by protein kinase A. Science *276*, 791-794.

Tong, J., Hannan, F., Zhu, Y., Bernards, A., Zhong, Y. (2002). Neurofibromin regulates G protein-stimulated adenylyl cyclase activity. Nature Neurosci. *5,* 95-96.

Yager, J., Richards, S,, Hekmat-Scafe, D.S., Hurd, D.D., Sundaresan, V., Caprette, D.R., Saxton, W.M., Carlson, J.R., Stern M (2001) Control of *Drosophila* perineurial glial growth by interacting neurotransmitter-mediated signaling pathways. Proc Natl Acad Sci USA *98,* 10445-10450.

APPENDIX

1) Abstract of presentation to the NNFF Consortium on NF1 and NF2, entitled " Evidence that PI3 Kinase mediates the effects of Ras on perineurial glial growth in Drosophila peripheral nerves" (Aspen, CO, May, 2004).

2) Abstract of presentation to the Drosophila research conference entitled " FOXO mediates the nonautonomous effects of Ras and PI3 Kinase on peripheral nerve growth", by William Lavery and Michael Stern (March, 2006, Houston, TX)

3) Abstract of presentation to the NNFF Consortium on NF1 and NF2 entitled " FOXO mediates the nonautonomous effects of Ras and PI3 Kinase on peripheral nerve growth", by William Lavery, Michael Stern (June, 2006, Aspen, CO).

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5) Abstract of presentation to the NNFF Consortium on NF1 and NF2 entitled " Ral, but not Raf, enhances the nonautonomous effects of PI3K on perineurial glial growth in the fly peripheral nerve" by William Lavery and Michael Stern (June, 2007, Park City, UT).

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7) Manuscript by Lavery, W., Hall, V., Yager, J.C., Rottgers, A., Wells, M.C. and Stern, M. (2007). Phosphatidyl inositol 3-Kinase and Akt nonautonomously promote perineurial glial growth in Drosophila peripheral nerves. *J. Neurosci* **27:** 279-288.

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# ABSTRACT FORM

TOPIC: Signaling pathways in NF and TSC

TITLE: Evidence that PI3 Kinase mediates the effects of Ras on perineurial glial growth in Drosophila peripheral nerves

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Drosophila peripheral nerves comprise a layer of motor and sensory axons, wrapped by an inner peripheral glia (analogous to the mammalian Schwann cell) and an outer perineurial glia (analogous to the mammalian perineurium). We have been using these nerves as an assay platform to test the effects of mutations and transgenes on perineurial glial growth. It was previously shown that perineurial glial growth in third instar larval nerves is regulated by a number of genes including *push*, which encodes a large $Zn^{2+}$-finger-containing protein, *amn*, which encodes a putative neuropeptide related to PACAP, and *NF1*. We found that expression of the constitutively active $Ras^{V12}$ transgene specifically in peripheral glia increased growth within the perineurial glia. This result demonstrates that Ras activity is sufficient to promote perineurial glial growth, and that Ras can act cell nonautonomously. Surprisingly, we found that the $NF1^{P2}$ null mutation suppresses these effects of $Ras^{V12}$, suggesting that *NF1* has a relevant activity that promotes, rather than inhibits, perineurial glial growth. The possibility that activation of adenylate cyclase represents this second activity is supported by the observation that expression within peripheral glia of any of three genes expected to increase protein kinase A (PKA) activity (a constitutively active PKA, the *amn*-encoded PACAP-like neuropeptide, or a constitutively active $G_\alpha s$) strongly enhances the growth promoting effects elicited by $Ras^{V12}$ alone. These results are consistent with the possibility that a signalling pathway from the Amn neuropeptide through $G_\alpha s$, Neurofibromin, and PKA strongly potentiates the effectiveness of constitutive Ras activity on perineurial glial growth.

To identify the downstream components that mediate the effects of Ras, we tested the effects of constitutively active *Raf* and *PI3 Kinase* transgenes on perineurial glial growth. We found that expression of a constitutively active *PI3 Kinase,* but not a constitutively active *Raf*, strongly increased perineurial glial growth, suggesting the possibility that PI3 Kinase is an important mediator of the growth-promoting effects of Ras in peripheral nerves.

FOXO mediates the nonautonomous effects of Ras and PI3 Kinase on peripheral nerve growth. William E. Lavery and Michael Stern
Department of Biochemistry and Cell Biology, Rice University, Houston, TX

**Drosophila peripheral nerves, structured similarly to their mammalian counterparts, comprise a layer of motor and sensory axons, wrapped by an inner peripheral glia (analogous to the mammalian Schwann cell) and an outer perineurial glia (analogous to the mammalian perineurium). We found that expression specifically within the peripheral glia of the constitutively active *Ras$^{V12}$* increases growth of the perineurial glial layer. This nonautonomous effect of Ras$^{V12}$ is mediated by activation of the downstream effector PI3 Kinase (PI3K) because expression within the peripheral glia of the activated *PI3K-CAAX* also increases perineurial glial growth, and because the growth-promoting effects of *Ras$^{V12}$* are suppressed by loss of function mutations in *PI3K* or by co-expression within the peripheral glia of the dominant-negative *PI3K$^{D954A}$*. The nonautonomous, growth-promoting effects of PI3K-CAAX are suppressed in a dose-dependent manner by loss of function mutations in *Akt*, the kinase downstream of *PI3K*, and are enhanced by co-expression within the peripheral glia of an *Akt$^{+}$* transgene. These observations suggest that PI3K exerts its effects via activation Akt. Finally, we show that the growth-promoting effects of *PI3K-CAAX* are suppressed by co-expression within the peripheral glia of *FOXO$^{+}$*, a transcription factor that is inhibited by Akt-dependent phosphorylation. We conclude that Ras-PI3K-Akt activity in the peripheral glia promotes growth of the perineurial glia by inhibiting FOXO. In mammalian peripheral nerves, the Schwann cell releases several growth factors that can affect the proliferative and migratory properties of neighbors. Some of these factors are oversecreted in Schwann cells defective in *Nf1*, which encodes the Ras-GTPase activator Neurofibromin and is the gene responsible for the disease type 1 Neurofibromatosis. Our results raise the possibility that peripheral nerve tumor formation in individuals with Neurofibromatosis might result at least in part from a Ras-PI3K-Akt-dependent inhibition of mammalian FOXO within Schwann cells.**

## Abstract

**FOXO mediates the nonautonomous effects of Ras and PI3 Kinase on peripheral nerve growth.** William Lavery, Michael Stern. Biochemistry and Cell Biology, Rice University, Houston, TX.

Drosophila peripheral nerves, structured similarly to their mammalian counterparts, comprise a layer of motor and sensory axons, wrapped by an inner peripheral glia (analogous to the mammalian Schwann cell) and an outer perineurial glia (analogous to the mammalian perineurium). We found that expression specifically within the peripheral glia of the constitutively active $Ras^{V12}$ increases growth of the perineurial glial layer. This nonautonomous effect of RasV12 is mediated by activation of the downstream effector PI3 Kinase (PI3K) because expression within the peripheral glia of the activated *PI3K-CAAX* also increases perineurial glial growth, and because the growth-promoting effects of $Ras^{V12}$ are suppressed by loss of function mutations in PI3K or by co-expression within the peripheral glia of the dominant-negative $PI3K^{D954A}$. The nonautonomous, growth-promoting effects of PI3K-CAAX are suppressed in a dose-dependent manner by loss of function mutations in *Akt*, the kinase downstream of *PI3K*, and are enhanced by co-expression within the peripheral glia of an $Akt^{+}$ transgene. These observations suggest that PI3K exerts its effects via activation of Akt. Finally, we show that the growth-promoting effects of *PI3K-CAAX* are suppressed by co-expression within the peripheral glia of $FOXO^{+}$, a transcription factor that is inhibited by Akt-dependent phosphorylation. We conclude that Ras-PI3K-Akt activity in the peripheral glia promotes growth of the perineurial glia by inhibiting FOXO. In mammalian peripheral nerves, the Schwann cell releases several growth factors that can affect the proliferative and migratory properties of neighbors. Some of these factors are oversecreted in Schwann cells defective in *Nf1*. Our results raise the possibility that neurofibromas might be caused at least in part by a Ras-PI3K-Akt-dependent inhibition of FOXO within Schwann cells.

*PI3K regulates neuronal excitability and axonal growth and arborization via distinct effector pathways.* /Eric Howlett, William Lavery, Michael Stern./ Biochemistry & Cell Biology, Rice University, Houston, TX.

The phosphatidylinositol 3-kinase (PI3K)/AKT pathway controls cellular survival and growth and has been implicated as a contributor to a wide variety of cancers. In the mouse CNS, activation of this pathway through loss of function mutations in the gene encoding PTEN, the phosphatase that opposes the effects of PI3K, results in increased neuronal arborization and neuronal hypertrophy; this system has been proposed to be a model for autism. PI3K has also been shown to induce synaptogenesis in Drosophila at both the larval neuromuscular junction (NMJ) and in the adult brain (Martín-Peña et al. J. Neurosci., Oct. 4, 2006. 26(40):10199-10208). Here we show that PI3K regulates motor axon diameter in the larval peripheral nerve: activation of this pathway via transgene expression increases axon diameter, whereas suppression of this pathway confers the opposite effect. Additionally, altered PI3K activity in motor neurons causes electrophysiological defects at the larval NMJ. In particular, activation of PI3K decreases neuronal excitability and both spontaneous and evoked transmitter release, whereas inhibition of PI3K confers the opposite phenotypes. Although effects of PI3K on neuronal growth have been previously shown to be mediated by the Tor pathway, we have found that the effects of PI3K on neuronal activity appear to be mediated by the transcription factor FOXO, which negatively regulates PI3K-induced transcription, and is inhibited by Akt-dependent phosphorylation. In particular, overexpression of FOXO in motor neurons increases neuronal excitability and partially suppresses the decrease in excitability conferred by PI3K. Our results suggest that the effects of PI3K on growth and activity are mediated by distinct effector pathways. These results provide a previously uncharacterized role for PI3K in regulating the relative excitability of neurons in vivo.

William Lavery
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Ral, but not Raf, enhances the nonautonomous effects of PI3K on perineurial glial growth in the fly peripheral nerve.

Drosophila peripheral nerves, structured similarly to their mammalian counterparts, comprise a layer of motor and sensory axons, wrapped by an inner peripheral glia (analogous to the mammalian Schwann cell) and an outer perineurial glia (analogous to the mammalian perineurium). We recently reported (Lavery et al., 2007) that expression specifically within the peripheral glia of the constitutively active $Ras^{V12}$ increases growth of the perineurial glial layer, and that this cell nonautonomous effect occurs through PI3-kinase (PI3K) and Akt-dependent inhibition of the transcription factor FOXO. Here we report that the growth-promoting effects of *PI3K-CAAX* are suppressed in flies expressing hypomorphic loss of function *Ras* alleles $Ras^{12A}/Ras^{e2F}$. Surprisingly, Ras is required in peripheral glia for this effect: expression of the dominant-negative $Ras^{N17}$ specifically in the peripheral glia also suppresses the effects of *PI3K-CAAX*, suggesting that Ras-mediated perineurial glial growth activation requires a downstream effector in addition to PI3K. In an effort to identify this downstream effector, we expressed within the peripheral glia gain-of-function or dominant-negative forms of *Raf*, either in an otherwise wildtype background or in combination with *PI3K-CAAX.*

We found that expression of these transgenes conferred only minor changes in perineurial glial thickness. Similarly, expression of the constitutively active $Ral^{V20}$ in peripheral glia in an otherwise wildtype background conferred no change in perineurial glial thickness. However, co-expression within the peripheral glia of *PI3K-CAAX* and $RAl^{V20}$ significantly enhanced the PI3K-induced growth effect on the perineurial glia. Therefore, we hypothesize that Ral in combination with PI3K activates perineurial glial growth nonautonomously.

**PI3K regulates neuronal excitability and axonal growth and arborization via distinct effector pathways.** *Eric Howlett, Curtis Lin, William Lavery, Michael Stern.*
Biochemistry & Cell Biology, Rice University, Houston, TX.

The phosphatidylinositol 3-kinase (PI3K)/AKT pathway controls cellular survival and growth and has been implicated as a contributor to a wide variety of cancers. In the mouse CNS, activation of this pathway through loss of function mutations in the gene encoding PTEN, the phosphatase that opposes the effects of PI3K, results in increased neuronal arborization and neuronal hypertrophy; this system has been proposed to be a model for autism. PI3K has also been shown to induce synaptogenesis in Drosophila at both the larval neuromuscular junction (NMJ) and in the adult brain (Martín-Peña et al. J. Neurosci., Oct. 4, 2006. 26(40):10199-10208). Here we show that PI3K regulates motor axon diameter in the larval peripheral nerve: activation of this pathway via transgene expression increases axon diameter, whereas suppression of this pathway confers the opposite effect. Additionally, altered PI3K activity in motor neurons causes electrophysiological defects at the larval NMJ. In particular, activation of PI3K decreases neuronal excitability and both spontaneous and evoked transmitter release, whereas inhibition of PI3K confers the opposite phenotypes. Although effects of PI3K on neuronal growth have been previously shown to be mediated by the Tor pathway, we have found that the effects of PI3K on neuronal activity appear to be mediated by the transcription factor FOXO, which negatively regulates PI3K-induced transcription, and is inhibited by Akt-dependent phosphorylation. In particular, overexpression of FOXO in motor neurons increases neuronal excitability and partially suppresses the decrease in excitability conferred by PI3K. Our results suggest that the effects of PI3K on growth and activity are mediated by distinct effector pathways. These results provide a previously uncharacterized role for PI3K in regulating the relative excitability of neurons in vivo. In addition, we have observed that alterations in the Drosophila metabotropic glutamate receptor (DmGluRA) effect both neuronal excitability and synaptogenesis in a manner similar to what we observe with PI3K, confirming results published by Bogdanik et al. (J. Neurosci., Oct. 13, 2004. 24(41):9105-16). Since it has been demonstrated that mGlur can activate PI3K via the HOMER/PIKE scaffolding complex, this raises the possibility that glutamate released at the NMJ could feed back onto mGlur, thus activating PI3K in an inhibitory feedback loop regulating both growth and excitability at the nerve terminal.

Cellular/Molecular

# Phosphatidylinositol 3-Kinase and Akt Nonautonomously Promote Perineurial Glial Growth in *Drosophila* Peripheral Nerves

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***Drosophila*** **peripheral nerves, structured similarly to their mammalian counterparts, comprise a layer of motor and sensory axons wrapped by an inner peripheral glia (analogous to the mammalian Schwann cell) and an outer perineurial glia (analogous to the mammalian perineurium). Growth and proliferation within mammalian peripheral nerves are increased by Ras pathway activation: loss-of-function mutations in *Nf1*, which encodes the Ras inhibitor neurofibromin, cause the human genetic disorder neurofibromatosis, which is characterized by formation of neurofibromas (tumors of peripheral nerves). However, the signaling pathways that control nerve growth downstream of Ras remain incompletely characterized. Here we show that expression specifically within the *Drosophila* peripheral glia of the constitutively active $Ras^{V12}$ increases perineurial glial thickness. Using chromosomal loss-of-function mutations and transgenes encoding dominant-negative and constitutively active proteins, we show that this nonautonomous effect of $Ras^{V12}$ is mediated by the Ras effector phosphatidylinositol 3-kinase (PI3K) and its downstream kinase Akt. We also show that the nonautonomous, growth-promoting effects of activated PI3K are suppressed by coexpression within the peripheral glia of $FOXO^{+}$ (forkhead box O) a transcription factor inhibited by Akt-dependent phosphorylation. We suggest that Ras–PI3K–Akt activity in the peripheral glia promotes growth of the perineurial glia by inhibiting FOXO. In mammalian peripheral nerves, the Schwann cell releases several growth factors that affect the proliferative properties of neighbors. Some of these factors are oversecreted in *Nf1* mutants. Our results raise the possibility that neurofibroma formation in individuals with neurofibromatosis might result in part from a Ras–PI3K–Akt-dependent inhibition of FOXO within Schwann cells.**

*Key words:* **neurofibromatosis; Ras; FOXO; cell growth; cell nonautonomy; Schwann cell**

## Introduction

Peripheral nerves in both *Drosophila* and mammals contain an inner layer of motor and sensory axons surrounded by an inner peripheral glial layer (termed the Schwann cell in mammals) and an outer, mesodermally derived perineurial glia (termed the perineurium in mammals). Proper growth, development, and function of peripheral nerves require intercellular signaling among the cell types present. For example, formation of the perineurial sheath requires Desert Hedgehog secretion from Schwann cells (Parmantier et al., 1999). In addition, neurons and glia interact reciprocally to regulate function, at least in part through the release of, and response to, small molecule neurotransmitters (Colomar and Robitaille, 2004; Yuan and Ganetzky, 1999).

Individuals with the autosomal-dominant genetic disorder of neurofibromatosis, which is caused by mutations in *Nf1* (for review, see Cichowski and Jacks, 2001), form peripheral nerve tumors called neurofibromas at high frequency. Neurofibromas are thought to arise in individuals heterozygous for *Nf1* after spontaneous loss of the $Nf1^{+}$ allele within Schwann cells (Kluwe et al., 1999; Serra et al., 2000). *NF1* encodes a Ras GTPase activator and thus negatively regulates Ras. Although at least some of the growth deficits of $Nf1^{-}$ cells result from Ras hyperactivation, the Ras effector pathways mediating the various growth defects have not been fully characterized. It was reported recently that phosphatidylinositol 3-kinase (PI3K), Akt, and the Akt-dependent kinase Tor (target of rapamycin) are hyperactivated in *Nf1*-deficient mouse or human cells and that this activation was required for proliferation of tumor cells in culture (Dasgupta et al., 2005; Johannessen et al., 2005). These results are consistent with the well established role for the PI3K–Tor pathway in autonomous growth control (Hay and Sonenberg, 2004). However, there is much evidence that neurofibroma formation requires Schwann cell nonautonomous pathways (Sherman et al., 2000; Yang et al., 2003). For example, neurofibromas are heterogeneous at the cellular level and contain cell types that are not clonally related (i.e., Schwann cells and fibroblasts). This observation raises the possibility that neurofibroma formation requires the $Nf1^{-}$-dependent oversecretion of growth factors that

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increase the proliferation of heterozygous neighbors (Yang et al., 2003). The identity of the pathway(s) regulating nonautonomous growth has not been elucidated.

Here we use the *Drosophila* peripheral nerve to identify molecules acting within the peripheral glia that regulate growth nonautonomously. We find that expression of constitutively active $Ras^{V12}$ specifically within the peripheral glia increases perineurial glial thickness. We also show that this nonautonomous, growth-activating effect is mediated by PI3K and Akt: PI3K and Akt activity within the peripheral glia are both necessary and sufficient to promote nonautonomous growth. Finally, we report that peripheral glial overexpression of *FOXO* (forkhead box O), which encodes a transcription factor inhibited by Akt-dependent phosphorylation and which antagonizes PI3K–Akt-dependent gene expression (Puig et al., 2003), suppresses the growth-promoting effects of activated PI3K. We conclude that the effect of Ras activity within the peripheral glia on perineurial glial growth is mediated by PI3K and Akt and suggest that this pathway promotes nonautonomous growth by inhibiting FOXO.

## Materials and Methods

Drosophila *stocks, mutations, and crosses.* Gliotactin (*gli*)–*Gal4* and *MZ709* express *Gal4* in peripheral glia (Ito et al., 1995; Auld et al., 1995; Leiserson et al., 2000; Sepp and Auld, 1999) and were provided by Vanessa Auld (University of British Columbia, Vancouver, British Columbia, Canada) and Kei Ito (National Institute for Basic Biology, Okazaki, Japan), respectively; upstream activating sequence (*UAS*)–*PI3K–CAAX* and *UAS–*$PI3K^{D954A}$ express a constitutively active and dominant-negative PI3K, respectively, under the transcriptional control of *Gal4* (Leevers et al., 1996) and were provided by Sally Leevers (Cancer Research Institute, London, UK); flies bearing *UAS–*$Ras^{V12}$ (strong) on chromosome III, *UAS–*$Ras^{+}$ (Lee et al., 1996; Karim and Rubin, 1998), *UAS–*$Raf^{F179}$ (Brand and Perrimon, 1994), *UAS*–green fluorescent protein *(GFP)* nuclear localization signal *(nls)* (Shiga et al., 1996), and $Akt^{4226}$ (Perrimon et al., 1996) were provided by the Bloomington *Drosophila* Stock Center (University of Indiana, Bloomington, IN). Two independent *UAS–Akt* transgenes (A. Park, personal communication to FlyBase) were provided by the Bloomington *Drosophila* Stock Center via Exelixis (South San Francisco, CA). *UAS–*$Ras^{V12}$ (weak) on chromosome II (Karim and Rubin, 1998) was provided by Andreas Bergmann (M. D. Anderson Cancer Research Center, Houston, TX). *UAS–*$Ral^{V20}$ (Sawamoto et al., 1999) was provided by Hideyuki Okano (Tokyo, Japan). Two independent *UAS–*$FOXO^{+}$ transgenes (Junger et al., 2003; Hwangbo et al., 2004) were provided by Marc Tatar (Providence, RI). Flies bearing two loss-of-function alleles of *PI3K*: $PI3K^{2H1}$ and $PI3K^{A}$ (Halfar et al., 2001), provided by Ernst Hafen (Zurich, Switzerland).

Standard *Drosophila* genetics techniques were used to establish the fly stocks and perform the crosses used in the experiments described. Because the *PI3K* and *Akt* loss-of-function alleles used confer either lethality or greatly reduced viability when homozygous, these alleles were maintained with balancers carrying the tubby *Tb* dominant marker, which can be scored in larvae. Third-instar larvae carrying the *Akt* or *PI3K* mutant alleles on both chromosomes, to be analyzed with electron microscopy, were recognized by their non-tubby appearance. For all experiments using either *Gal4* or *UAS* transgenes, the appropriate larvae were obtained after a cross of the *Gal4*-containing fly line to the *UAS*-containing fly line. Because *UAS–PI3K–CAAX* is located on the X chromosome, only female larvae heterozygous for these transgenes were analyzed. In all cases, larvae bearing the *Gal4* driver alone or the *UAS*-driven transgene alone were generated in parallel to the experimental larvae and used as controls.

*Transmission electron microscopy.* Larvae were grown to the wandering third-instar stage in uncrowded half-pint bottles at room temperature (22–23°C). Larvae were collected only during the first and second days after the initial third-instar larvae appeared. The dissections, fixations, and stainings were performed as described previously (Yager et al., 2001). Perineurial glial thickness was measured from the edge of the nerve to the axon-containing lumen and averaged from eight measurements made 12:00, 3:00, 6:00, and 9:00 and four positions in between. Measurements were not taken at positions in nerves in which a perineurial glial nucleus was encountered.

*Fluorescence microscopy.* Larvae were grown to the wandering third-instar stage as described above. These larvae were dissected with the protocol used for electron microscopy, except PBS was used for dissections. Dissected larvae were fixed in PBS containing 5% formaldehyde and 0.1% Triton X-100 for 15 min. Ventral ganglia and nerves were removed and placed in Vectashield (H-1000; Vector Laboratories, Burlingame, CA) containing a 1:1000 dilution of Hoechst stain (H-3570; Invitrogen, Carlsbad, CA). Nuclei were visualized with 4′,6′-diamidino-2-phenylindole and GFP filters on an Axioplan 2 epifluorescence microscope (Zeiss, Oberkochen, Germany) using MetaMorph software for micrograph acquisition (Molecular Devices, Palo Alto, CA).

## Results

### *gli–Gal4* and *MZ709*: two *Gal4* drivers that express in the peripheral glia but not the perineurial glia.

*Drosophila* peripheral nerves contain a layer of ~80 motor and sensory axons, wrapped by an inner peripheral glia, which forms the blood–nerve barrier (Auld et al., 1995) and an outer, mesodermally derived perineurial glia (Edwards et al., 1993). A transmission electron micrograph (TEM) of a peripheral nerve cross section is shown in Figure 1*A*. Each peripheral nerve contains approximately eight peripheral glial nuclei (Sepp et al., 2000). In addition, each mm of peripheral nerve contains ~20 perineurial glial nuclei (W. Lavery and M. Stern, unpublished observations).

To evaluate the role of Ras signaling in nonautonomous growth control within peripheral nerves, we used the *Gal4/UAS* system (Brand and Perrimon, 1993) to express wild-type and mutant transgenes specifically within the peripheral glia. Two *Gal4* drivers, *gli–Gal4* and *MZ709*, were reported to express in the peripheral glia but not the neurons of peripheral nerves (Ito et al., 1995; Sepp and Auld, 1999; Leiserson et al., 2000; Sepp et al., 2000). The *gli–Gal4* driver is a particularly well characterized marker for peripheral glia. *gli–Gal4* was generated via gene conversion from a *gli–lacZ* enhancer trap line (Auld et al., 1995; Sepp and Auld, 1999), which was reported to express specifically in peripheral glia, exit glia, and some midline glia. The *gli–Gal4* driver was used to study peripheral glial dynamics during embryonic peripheral nerve development. This driver was also used to study peripheral glial anatomy during larval growth and at the mature third-instar larval neuromuscular junction and peripheral sensory structures (Sepp et al., 2000). These studies confirmed that *gli–Gal4* is expressed in peripheral glia but not motor and sensory neurons.

To confirm that *gli–Gal4* and *MZ709* do not express *Gal4* in the perineurial glia, we visualized the expression pattern of these drivers within peripheral nerves via induced expression of a nuclear-localized GFP. We also visualized the total complement of peripheral nerve nuclei (peripheral and perineurial glial) via the Hoechst DNA dye. As shown in Figure 1, *B* and *D*, there are ~20 nuclei per millimeter of peripheral nerve. Most of these are perineurial glial nuclei, whereas a few are peripheral glial nuclei. If *gli–Gal4* and *MZ709* express in the perineurial glia as well as peripheral glia, then we anticipate that, in *gli*>*GFP(nls)* and *MZ709*>*GFP(nls)*, most or all of these nuclei would contain GFP. In fact, as shown in Figure 1, *C* and *E*, we observe that only a few (presumably peripheral glial) nuclei from these larvae express GFP. Therefore, we conclude that neither *gli–Gal4* and *MZ709* expresses *Gal4* in the perineurial glia. We generally observe GFP in fewer than eight peripheral glial nuclei, which presumably results from cell-to-cell variability in *Gal4* expression levels, as

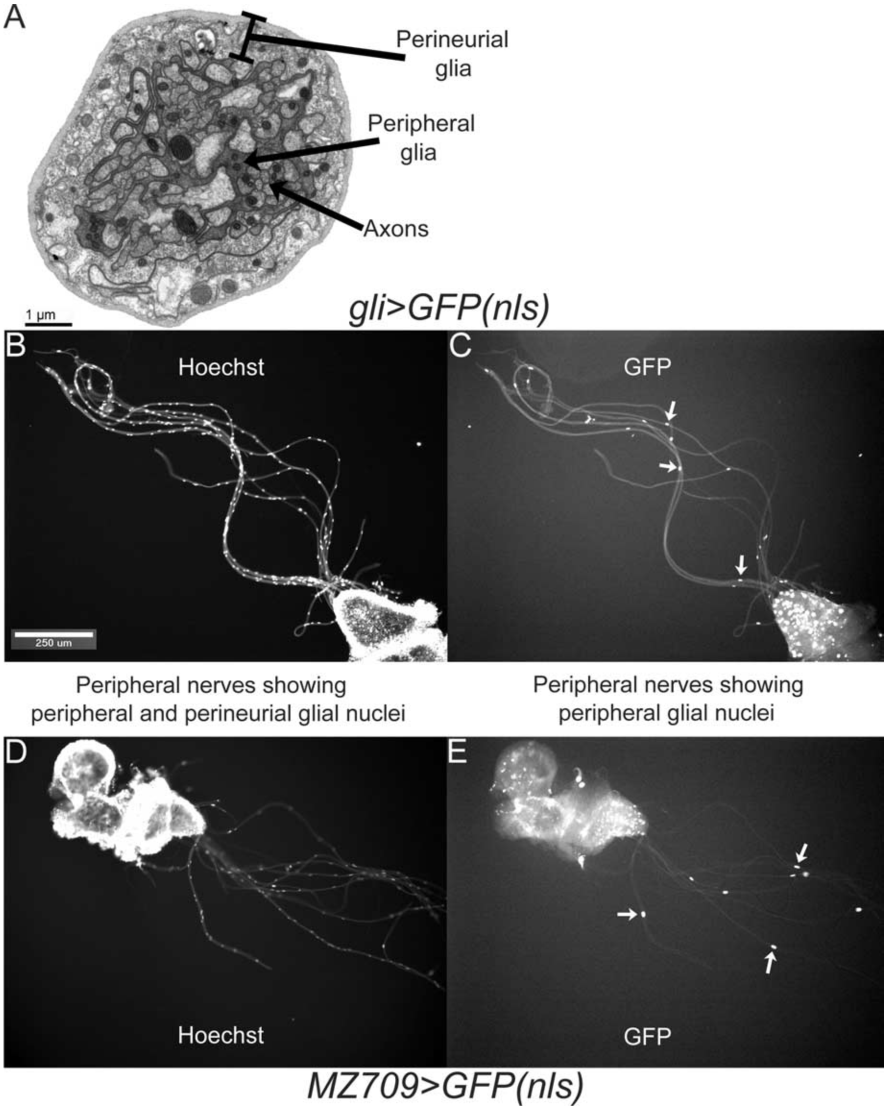



**Figure 1.** *gli–Gal4* and *MZ709* drivers are expressed in the peripheral glia but not the perineurial glia. ***A***, TEM of a cross section of a *gli*>*Ras*$^+$ third-instar larval peripheral nerve of wild-type thickness. The cell types present are indicated. ***B***, ***C***, Epifluorescence images of third-instar larval peripheral nerves from *gli*>*GFP(nls)* visualized for Hoechst and GFP, respectively. All nuclei (peripheral glial and perineurial glial) are visualized with the Hoechst stain (***B***), whereas only a few nuclei (presumed to be peripheral glial), some marked with arrows, are visualized with GFP (***C***). ***D***, ***E***, Same as ***B*** and ***C*** except that *MZ709*>*GFP(nls)* larvae were visualized. These observations demonstrate that *gli–Gal4* and *MZ709* are not expressed in perineurial glia.

was reported previously for peripheral glia (Sepp et al., 2001). We also observed that each driver also expresses *Gal4* within certain cells of the ventral ganglion (Fig. 1).

### Expression of the constitutively active *Ras*$^{V12}$ allele in peripheral glia increases perineurial glial growth

In both mice and humans, neurofibroma formation appears to occur only when the Schwann cell component of the peripheral nerve is homozygous for Nf1$^-$ (Zhu et al., 2002; Kluwe et al., 1999). This observation suggests that activated Ras within Schwann cells is necessary for neurofibroma formation. To test the effects of activating Ras within *Drosophila* peripheral glia (analogous to the mammalian Schwann cell), we used the *gli–Gal4* driver to express *Ras*$^+$ or the constitutively active *Ras*$^{V12}$ (Bourne et al., 1991; Lee et al., 1996; Karim and Rubin, 1998) specifically in the peripheral glia. We found that larvae bearing *gli–Gal4* and either of two *UAS–Ras*$^{V12}$ transgenes exhibited a thickened perineurial glia. The thickness observed, 2.1–2.3 $\mu$m, was significantly (~50%) greater than the value observed in larvae carrying *gli–Gal4* or *UAS–Ras*$^{V12}$ alone or *gli*>*Ras*$^+$ (Fig. 2). We conclude that Ras activation specifically within the peripheral glia is sufficient to promote perineurial glial growth. We also found that *gli–Gal4*-driven coexpression of both *UAS–Ras*$^{V12}$ transgenes does not cause an additional increase in perineurial glial thickness: perineurial glial thickness in larvae expressing both transgenes is the same as in larvae expressing either transgene alone (Fig. 2). This observation suggests that, in *gli*>*Ras*$^{V12}$ larvae, *Ras*$^{V12}$ levels are not limiting for promoting perineurial glial growth. To rule out the possibility that the presence of two transgenes decreased expression of both via titration of *Gal4*, we measured perineurial glial thickness in larvae coexpressing *Ras*$^{V12}$ with an indifferent transgene (*GFP*). We found that this coexpression did not suppress the growth-promoting effects of *Ras*$^{V12}$ (Fig. 2), suggesting that the presence of a second *UAS*-driven transgene does not significantly affect expression of the first.

### PI3K activation in the peripheral glia is sufficient to increase perineurial glial growth

Activated Ras activates a number of downstream molecules, including Raf, PI3K, and the guanine nucleotide exchange factor for the Ral GTPase (Kolch et al., 1991; Rodriguez-Viciana et al., 1994; Hofer et al., 1994). To identify the effector(s) responsible for transducing the nonautonomous growth activation conferred by *Ras*$^{V12}$, we expressed transgenes encoding the constitutively active *Raf*$^{F179}$, *PI3K–CAAX*, and *Ral*$^{V20}$ proteins (Brand and Perrimon, 1994; Leevers et al., 1996; Sawamoto et al., 1999) within peripheral glia using *gli–Gal4*. As shown in Figure 3*B*, we found that expression of *Raf*$^{F179}$ or *Ral*$^{V20}$ had no significant effect on perineurial glial thickness. However, expression of *PI3K–CAAX* increased perineurial glial thickness to ~3 $\mu$m (Fig. 3*A*,*B*). This thickness is significantly greater than both wild-type thickness and the increased thickness conferred by *Ras*$^{V12}$ expression. When *UAS–PI3K–CAAX* was expressed with a second peripheral glial driver, *MZ709* (Ito et al., 1995) (Fig. 1), perineurial glial thickness was increased to the same extent as with *gli–Gal4*. These results suggest that Ras exerts its nonautonomous effects on perineurial glial growth via activation of PI3K. The observation that *PI3K–CAAX* exerts a stronger effect than *Ras*$^{V12}$ might indicate that PI3K levels are limiting in peripheral glia to promote perineurial glial growth. In this view, transgene-induced overexpression of *PI3K–CAAX* overcomes this limitation and enables a more robust growth effect to be observed.

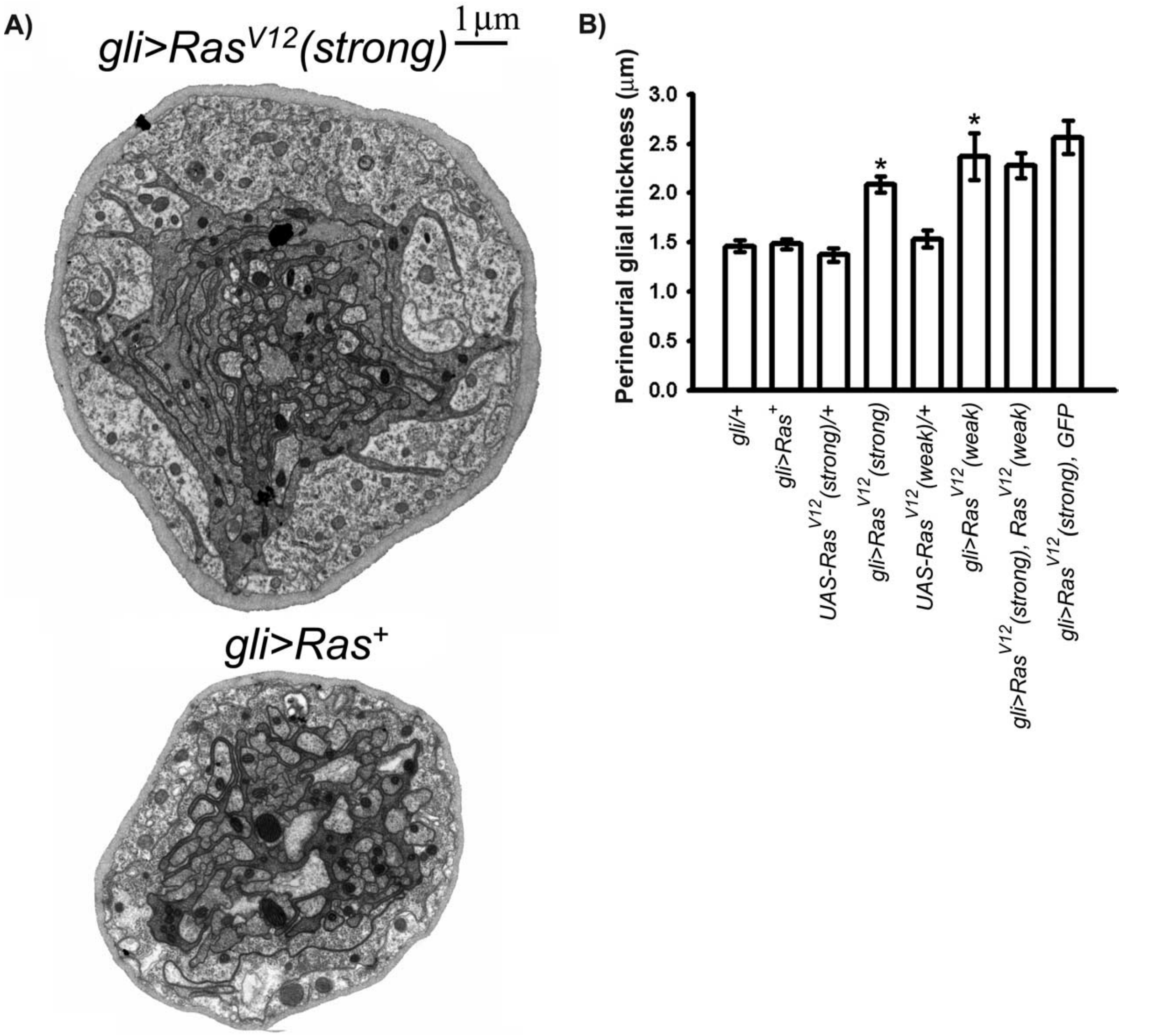
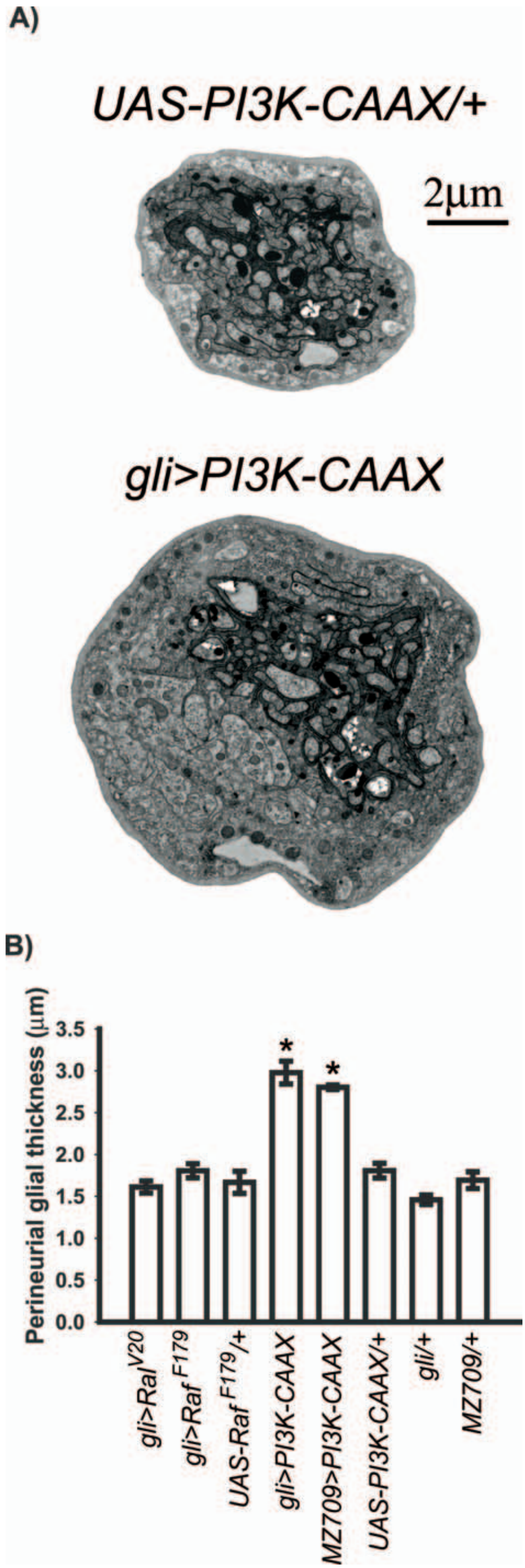



**Figure 2.** Expression of activated Ras in peripheral glia increases perineurial glial growth. ***A***, TEMs of cross sections from representative peripheral nerves of the indicated genotypes. The *gli*>*Ras*$^{+}$ nerve is the same nerve cross section shown Figure 1*A*. ***B***, Perineurial glial thickness (*y*-axis) from the indicated genotypes (*x*-axis). Means ± SEMs are indicated. One-way ANOVA and Scheffé's tests for multiple comparisons showed the following statistically significant differences, denoted by asterisks: *gli*>*Ras*$^{V12}$*(strong)* (2.08 ± 0.082 µm; $n = 78$), *gli*>*Ras*$^{V12}$*(weak)* (2.37 ± 0.236 µm; $n = 20$), *gli*>*Ras*$^{V12}$*(strong)*, *Ras*$^{V12}$*(weak)* (2.28 ± 0.130 µm; $n = 56$), and *gli*>*Ras*$^{V12}$*(strong)*, *GFP* (2.57 ± 0.17 µm; $n = 43$) versus *gli*>*Ras*$^{+}$ (1.48 ± 0.048 µm; $n = 99$), *gli–Gal4*/+ (1.46 ± 0.057 µm; $n = 60$), *UAS–Ras*$^{V12}$*(strong)*/+ (1.37 ± 0.068 µm; $n = 45$), and *UAS-Ras*$^{V12}$*(weak)*/+ (1.53 ± 0.088 µm; $n = 22$). For all such tests, $p < 0.0001$.

**PI3K activity in the peripheral glia is necessary to mediate the nonautonomous, growth-promoting effect of *Ras*$^{V12}$**

The results shown in Figure 3 demonstrate that PI3K activation in peripheral glia is sufficient to increase perineurial glial growth. To determine whether PI3K activity is necessary for the nonautonomous growth-promoting effects of *Ras*$^{V12}$, we introduced the heteroallelic *PI3K* loss-of-function combination *PI3K*$^{2H1}$/*PI3K*$^{A}$ (Halfar et al., 2001) into *gli*>*Ras*$^{V12}$ larvae. This mutant combination was chosen because it decreases PI3K activity sufficiently to confer phenotypes but retains sufficient activity to permit viability to the third-instar larval stage. We found that *PI3K*$^{2H1}$/*PI3K*$^{A}$ significantly suppressed the growth-promoting effects of *Ras*$^{V12}$ (Fig. 4), which demonstrates that PI3K activity is necessary for this effect. To determine whether PI3K activity is necessary in peripheral glia rather than the perineurial glia, we blocked PI3K activity specifically in the peripheral glia by coexpressing *Ras*$^{V12}$ with a transgene encoding the dominant-negative *PI3K*$^{D954A}$ (Leevers et al., 1996). We found that the peripheral–glial-specific expression of *PI3K*$^{D954A}$ blocked the growth-promoting effects of *Ras*$^{V12}$ (Fig. 4), suggesting that PI3K activity is required in the peripheral glia to promote perineurial glial growth. In contrast, as described above, coexpressing *Ras*$^{V12}$ with *GFP* did not suppress the growth-promoting effects of *Ras*$^{V12}$ (Fig. 2).

To confirm that *PI3K*$^{2H1}$/*PI3K*$^{A}$ suppressed the *Ras*$^{V12}$ phenotype by decreasing PI3K activity in the peripheral glia rather than the perineurial glia, we introduced *PI3K*$^{2H1}$/*PI3K*$^{A}$ into *gli*>*PI3K–CAAX* larvae. The extremely thick perineurial glia conferred by *PI3K–CAAX* was not significantly affected by the presence of *PI3K*$^{2H1}$/*PI3K*$^{A}$ (Fig. 4); thus, the perineurial glia in the *PI3K*$^{2H1}$/*PI3K*$^{A}$ mutant is fully competent to respond to growth-promoting signals from the peripheral glia, which



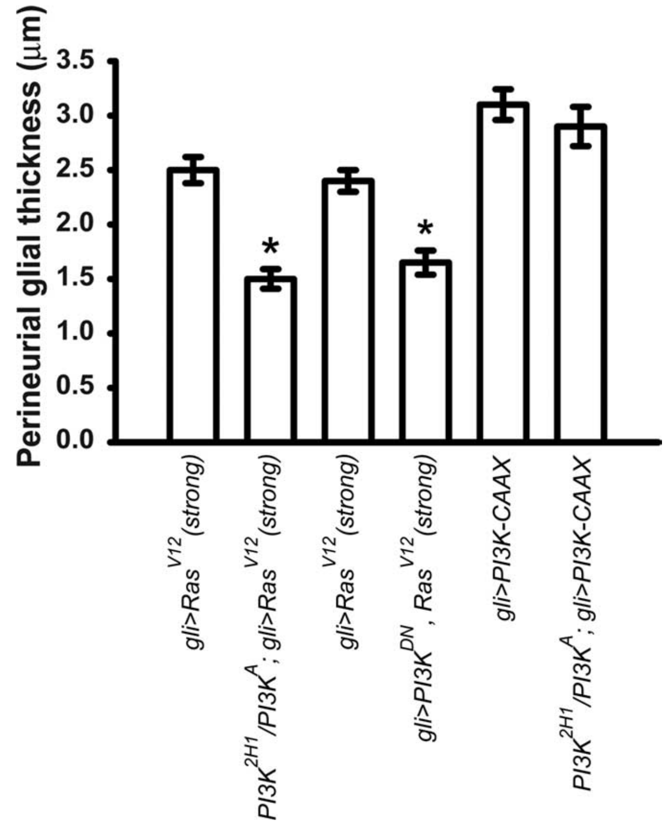




**Figure 4.** *Ras$^{V12}$* requires PI3K activity in the peripheral glia to increase perineurial glial growth. Histograms show perineurial glial thickness ( *y*-axis) from the indicated genotypes (*x*-axis). Means ± SEMs are indicated. The increase in perineurial glial thickness observed in *gli*>*Ras$^{V12}$* (lane 1) is significantly suppressed by the heteroallelic loss-of-function combination *PI3K$^{2H1}$/PI3K$^{A}$* (lane 2). The *gli*>*Ras$^{V12}$* larvae analyzed for lane 1 were obtained from the *PI3K$^{+}$* recombinants when *PI3K$^{A}$* was crossed onto *UAS–Ras$^{V12}$*, whereas the *PI3K$^{2H1}$/PI3K$^{A}$*; *gli*>*Ras$^{V12}$* larvae analyzed were obtained from the *PI3K$^{A}$* recombinants from this cross; thus, the values for the genotypes shown in lanes 1 and 2 are paired. The following pairwise combinations had statistically significant differences (two-tailed, unpaired *t* test) denoted by asterisks: *gli*>*Ras$^{V12}$(strong)* (lane 1; 2.46 ± 0.13 $\mu$m; $n = 50$) versus *PI3K$^{2H1}$/PI3K$^{A}$*; *gli*>*Ras$^{V12}$* (1.54 ± 0.05 $\mu$m; $n = 85$), $p < 0.0001$; for *gli*>*Ras$^{V12}$(strong)* (lane 3; 2.41 ± 0.111 $\mu$m; $n = 72$) versus *gli*>*Ras$^{V12}$(strong)*, *PI3K$^{D954A}$* (1.75 ± 0.08 $\mu$m; $n = 49$), $p < 0.0001$. In contrast, *gli*>*PI3K–CAAX* (3.1 ± 0.14 $\mu$m; $n = 53$) was not significantly different from *PI3K$^{2H1}$/PI3K$^{A}$*; *gli*>*PI3K–CAAX* (2.88 ± 0.35 $\mu$m; $n = 11$), $p = 0.43$

strongly suggests that the significant suppression of the *Ras$^{V12}$* growth phenotype by *PI3K$^{2H1}$/PI3K$^{A}$* results from loss of PI3K activity in the peripheral glia.

### The PI3K effector Akt mediates the nonautonomous effects of PI3K on perineurial glial growth

One PI3K effector is the protein kinase Akt (Scheid and Woodgett, 2001). Elevated PI3K activity promotes the ability of

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**Figure 3.** Peripheral glial activity of constitutively active PI3K, but not constitutively active Ral or Raf, is sufficient to increase perineurial glial growth. ***A***, TEMs of cross sections from representative peripheral nerves of the indicated genotypes. ***B***, Perineurial glial thickness ( *y*-axis) from the indicated genotypes (*x*-axis). Means ± SEMs are indicated. One-way ANOVA and Scheffé's tests for multiple comparisons showed the following statistically significant differences, denoted by asterisks: *gli*>*PI3K–CAAX* (2.98 ± 0.136 $\mu$m; $n = 76$) and *MZ709*>*PI3K–CAAX* (2.80 ± 0.263 $\mu$m; $n = 33$) versus *UAS–PI3K–CAAX/+* (1.81 ± 0.088 $\mu$m; $n = 59$), *gli–Gal4/+* (1.46 ± 0.057 $\mu$m; $n = 60$) and *MZ709/+* (1.69 ± 0.10 $\mu$m; $n = 27$). For all such tests, $p < 0.0001$. In contrast, *gli*>*Raf$^{F179}$* and *gli*>*Ral$^{V20}$* showed no significant increase in perineurial glial thickness.

the kinase PI3K-dependent kinase PDK1 to phosphorylate and activate Akt. To determine whether Akt activity was necessary for the growth-promoting effects of PI3K, we replaced either one or both copies of *Akt*$^+$ with the strong hypomorphic *Akt*$^{4226}$ allele (Perrimon et al., 1996) in *gli*>*PI3K–CAAX* larvae. We found that replacing one copy of *Akt*$^+$ moderately suppressed, and replacing both copies of *Akt*$^+$ profoundly suppressed, the effects of *PI3K–CAAX* (Fig. 5). These results demonstrate that Akt activity is required for the growth-promoting effects of PI3K. Akt activity can promote growth cell autonomously (Hay and Sonenberg, 2004). Thus, *Akt*$^{4226}$ could suppress the growth-promoting effects of *PI3K–CAAX* by decreasing Akt activity in either the peripheral or perineurial glia. To determine whether *Akt*$^+$ activity in the peripheral glia was sufficient to increase perineurial glial growth, we measured perineurial glial thickness in larvae expressing either of two *UAS–Akt*$^+$ transgenes driven by *gli–Gal4* and found no effect on the perineurial glia (Fig. 5). Because these *Akt*$^+$ transgenes encode wild-type Akt, which requires activation by the PI3K-dependent kinase PDK1, it was possible that this lack of effect might result from low endogenous PI3K activity in the peripheral glia, which would lead to inability to activate Akt. To test this possibility, we activated Akt in the peripheral glia by using *gli–Gal4* to co-overexpress *UAS–Akt*$^+$ with *UAS–PI3K–CAAX*. We found a striking increase in perineurial glial thickness in this genotype compared with larvae overexpressing *PI3K–CAAX* alone (Fig. 5; note that the *gli*>*PI3K–CAAX, Akt*$^+$ nerve pictured is an extreme nerve, not a typical nerve). This result suggests that, in the presence of activated PI3K, Akt levels within the peripheral glia become limiting for activating growth nonautonomously. In this view, increasing Akt levels by transgene expression serves to relieve this limitation and enable an additional increase in perineurial glial growth. We conclude that Akt activation in the peripheral glia is sufficient to increase perineurial glial growth.

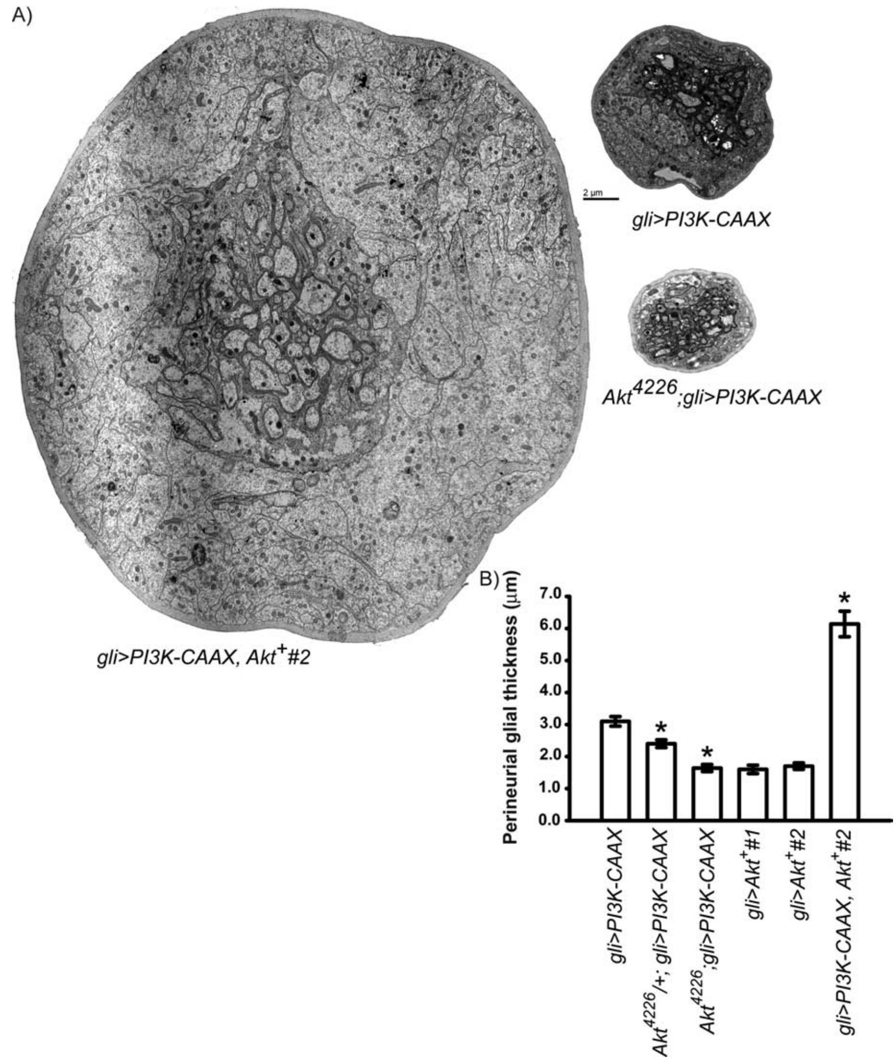



**Figure 5.** Akt activity in the peripheral glia is necessary and sufficient for PI3K-induced nonautomous growth activation. ***A***, TEMs of cross sections from peripheral nerves of the indicated genotypes. The *gli*>*PI3K–CAAX, Akt*$^+$*#2* nerve was too large to be photographed in a single electron micrograph, and thus a photo montage composed of four separate photographs is shown. This nerve is not typical and represents one of the larger nerves of this genotype. The same *gli*>*PI3K–CAAX* nerve from Figure 3 is shown for relative comparison with perineurial glial thickness of other genotypes. ***B***, Perineurial glial thickness ( *y*-axis) from the indicated genotypes (*x*-axis). Means ± SEMs are indicated. One-way ANOVA and Scheffé's tests for multiple comparisons showed the following statistically significant differences, denoted by asterisks: *gli*>*PI3K–CAAX* (2.98 ± 0.136 $\mu$m; $n = 76$) versus *Akt*$^{4226}$/+; *gli*>*PI3K–CAAX* (2.42 ± 0.16 $\mu$m; $n = 29$), $p = 0.02$, and versus *Akt*$^{4226}$/*Akt*$^{4226}$; *gli*>*PI3K–CAAX* (1.65 ± 0.42 $\mu$m; $n = 52$), $p < 0.0001$. Also, *gli*>*PI3K–CAAX, Akt*$^+$*#2* (6.14 ± 0.45 $\mu$m; $n = 28$) versus *gli*>*PI3K–CAAX*, *gli*>*Akt*$^+$*#1* (1.48 ± 0.057 $\mu$m; $n = 32$) and *gli*>*Akt*$^+$*#2* (1.52 ± 0.068 $\mu$m; $n = 22$), $p < 0.0001$. *UAS–Akt*$^+$*#1* and *UAS–Akt*$^+$*#2* are independent insertions of the same transgene.

In addition to the effect of *gli*>*PI3K–CAAX, Akt* on perineurial glial thickness, we observed a significant increase in thickness of the "axon bundle" (motor and sensory axons and peripheral glia) in this genotype. This increased thickness is attributable mostly to the presence of motor and sensory axons of increased diameter (Fig. 5). A more complete description of this phenotype will be presented in a future study. However, these observations suggest that extremely high levels of Akt activity can nonautonomously activate axonal growth as well as perineurial glial growth.

### FOXO overexpression suppresses the growth-promoting effects of PI3K

One Akt effector is the forkhead-box transcription factor FOXO. FOXO inhibits PI3K- and Akt-dependent gene expression; this activity is lost during phosphorylation by Akt, which causes phospho-FOXO to be excluded from the nucleus (Brunet et al., 1999). To test the possibility that PI3K and Akt activity increase perineurial glial growth by inhibiting FOXO, we coexpressed *PI3K–CAAX* and either of two *FOXO* transgenes (Hwangbo et al., 2004) within the peripheral glia. We found that expression of either *FOXO* transgene significantly suppressed the growth-promoting effects of *PI3K–CAAX* (Fig. 6). In contrast, when we coexpressed *PI3K–CAAX* with a neutral *UAS*-driven transgene (*UAS–GFP*), we did not observe significant suppression (Fig. 6).

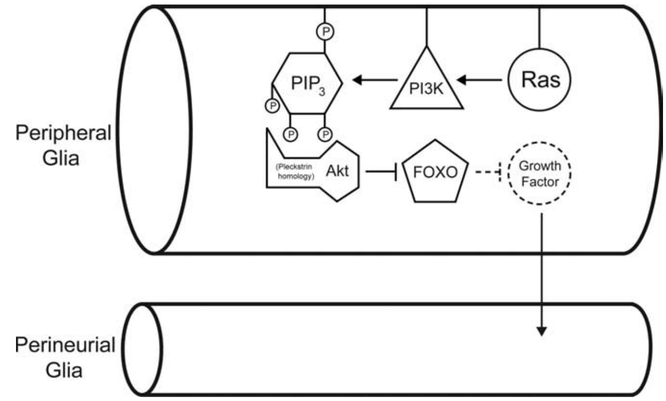


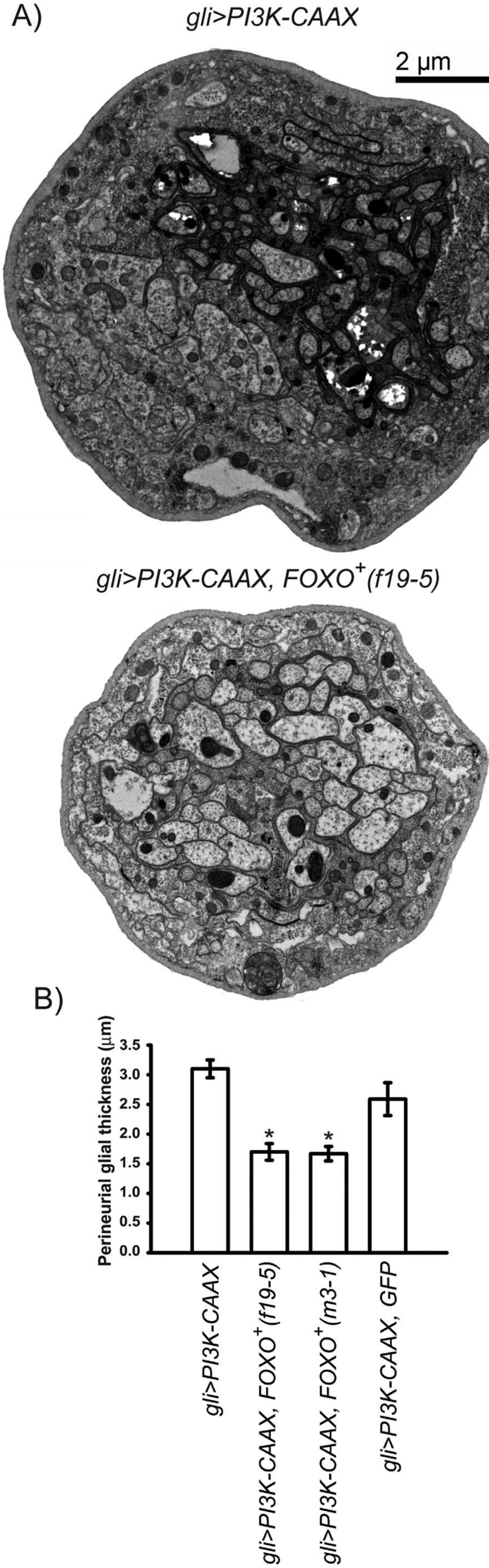

**Figure 7.** Model for the nonautonomous control of perineurial glial growth by the PI3K pathway. Peripheral and perineurial glial cells are indicated. The experimental evidence reported here demonstrates that Ras–PI3K–Akt activity in the peripheral glia increases growth of the perineurial glia and suggests that this activation occurs by the inhibition of FOXO (solid arrows and boxes). We hypothesize (dashed arrows and boxes) that FOXO directly or indirectly inhibits expression of a growth factor that activates perineurial glial cell growth. $PIP_3$, Phosphatidylinositol (3,4,5)-trisphosphate; P, phosphate.

Thus, *FOXO* overexpression suppresses the growth-promoting effects of PI3K.

Our studies provide new mechanistic insights into the nonautonomous growth-promoting effects of peripheral glia (Schwann cells) in peripheral nerves. Our results are completely consistent with the possibility that these nonautonomous effects are mediated by a pathway in which the negative regulation of growth by FOXO is inhibited by its Akt-dependent phosphorylation. FOXO might directly or indirectly repress transcription of growth factors that recruit the growth of neighbors.

## Discussion

We report the effects of altered activity of Ras and downstream effectors on growth within *Drosophila* peripheral nerves. We found that activating Ras specifically within the peripheral glia was sufficient to increase growth of the perineurial glia. In addition, we found that activating the Ras effector PI3K (Rodriguez-Viciana et al., 1994), but not Raf or Ral, within the peripheral glia was sufficient to increase perineurial glial growth and that inhibiting PI3K activity in the peripheral glia, but not perineurial glia, suppressed the growth-promoting effects of activated Ras. We also found that activity within the peripheral glia of the PI3K-activated kinase Akt (Franke et al., 1995; Scheid and Woodgett, 2001) was both necessary and sufficient to mediate the growth-promoting effects of PI3K. Finally, we found that overexpression within the peripheral glia of FOXO, the forkhead-box transcription factor that is phosphorylated and inactivated by Akt-dependent phosphorylation (Brunet et al., 1999), was sufficient to suppress the growth-promoting effects of PI3K. Together,




←

**Figure 6.** PI3kinase and Akt increase perineurial glial growth by inhibition of FOXO. ***A***, TEMs of cross sections from peripheral nerves of the indicated genotypes. The same *gli–PI3K–CAAX* nerve from Figure 3 is shown for relative comparison of perineurial glial thickness to *gli>PI3K–CAAX, FOXO⁺ (f19-5)* nerve. ***B***, Perineurial glial thickness ( *y*-axis) from the indicated genotypes (*x*-axis). Means ± SEMs are indicated. One-way ANOVA and Scheffé's tests for multiple comparisons showed the following statistically significant differences, denoted by asterisks: *gli>PI3K*–CAAX (2.98 ± 0.136 $\mu$m; $n = 76$) and *gli>PI3K–CAAX, GFP* (2.59 ± 0.28 $\mu$m; $n = 25$) versus *gli>PI3K–CAAX, $FOXO^+$ (f19-5)* (1.67 ± 0.086 $\mu$m; $n = 30$), $p < 0.004$, and versus *gli>PI3K–CAAX, $FOXO^+$ (m3-1)* (1.70 ± 0.098 $\mu$m; $n = 64$), $p < 0.01$. *UAS–$FOXO^+$ (f19-5)* and *UAS–$FOXO^+$ (m3-1)* are independent insertions of the same transgene.

these results suggest that Ras activity in the peripheral glia activates nonautonomous growth via the PI3K and Akt-dependent inhibition of FOXO (Fig. 7). This observation is consistent with the previous observations that *Nf1*$^{-}$ mouse Schwann cells oversecrete growth factor(s) that cause increased recruitment of mast cells into the peripheral nerve (Yang et al., 2003) and is consistent in part with the observation that the proliferation defects of *Nf1*$^{-}$ mutant mouse or human cells requires hyperactivation of Tor in a PI3K- and Akt-dependent manner (Dasgupta et al., 2005; Johannessen et al., 2005).

**Regulation of peripheral nerve growth by a neuron–glia signaling pathway**

Yager et al. (2001) reported that perineurial glial growth in *Drosophila* peripheral nerves is regulated by several genes. These genes include *Nf1*, which is the *Drosophila* ortholog of human *Nf1*, *push*, which is thought to encode an E3 ubiquitin ligase and two genes implicated in neurotransmitter signaling: *amnesiac*, which is thought to encode a neuropeptide similar to vertebrate pituitary adenylate cyclase-activating polypeptide (Feany and Quinn, 1995), and inebriated (*ine*), which encodes a member of the $Na^{+}/Cl^{-}$-dependent neurotransmitter transporter family (Soehnge et al., 1996). Some of these genes might regulate perineurial glial growth via the activity of Ras or PI3K within peripheral glia. For example, mutations in *push*, but not *ine*, enhance the perineurial glial growth phenotype of *Ras*$^{V12}$ expressed in peripheral glia (Lavery and Stern, unpublished observations). These observations are consistent with the possibility that the activity of *ine* regulates Ras-GTP levels within peripheral glia. In contrast, *push* might regulate PI3K in a Ras-independent manner or act in the perineurial glia to regulate sensitivity to peripheral glial growth factors. Additional experiments will be required to distinguish between these possibilities.

**Regulation of peripheral nerve growth by Schwann cell nonautonomous mechanisms**

There are several lines of evidence from mice and humans suggesting that cell nonautonomous growth regulation, as a consequence of intercellular signaling, underlies neurofibroma formation. First, although neurofibromas arise in individuals heterozygous for *Nf1*$^{-}$ after loss of *Nf1*$^{+}$ from cell(s) within peripheral nerves, neurofibromas are heterogeneous and contain cells that are not clonally related, such as Schwann cells, perineurial cells, and fibroblasts. These observations suggest that neurofibromas arise when a core of *Nf1*$^{-}$ cells cause overproliferation of their heterozygous neighbors via nonautonomous means. Second, neurofibroma formation in mice and humans requires a homozygous *Nf1* mutant genotype in Schwann cells but not other cells within the tumor (Kluwe et al., 1999; Zhu et al., 2002). Third, Ras-GTP levels in Schwann cells from the mouse *Nf1* knock-out mutant are uniformly elevated. In contrast, only a subset of Schwann cells from human neurofibromas exhibit elevated Ras- GTP levels (Sherman et al., 2000); these authors raised the possibility that this subset, but not other Schwann cells from the tumor, was homozygous for *Nf1*$^{-}$. In this view, these *Nf1*$^{-}$ cells recruited neighboring Schwann cells that were heterozygous for *Nf1*$^{-}$ into the tumor by nonautonomous means, such as by the excessive release of one or more growth factors. Fourth, Yang et al. (2003) demonstrated that *Nf1*$^{-}$ Schwann cells oversecrete the ligand for the c-Kit receptor. This oversecretion increased migration of mast cells into peripheral nerves and might be an essential step in neurofibroma formation. These Schwann cells also oversecrete additional factors whose physiological role remains unclear (Yang et al., 2003). The molecular mechanisms by which neurofibromin regulates the synthesis or release of these molecules remain incompletely understood. Our observations that Ras activity in the peripheral glia promotes growth nonautonomously via the PI3K- and Akt-dependent inhibition of FOXO might provide insights into the mechanisms by which peripheral nerve growth is regulated nonautonomously by the mammalian Schwann cell.

**Regulation of peripheral nerve growth by Ras effectors**

By hyperactivating Ras, *Nf1* mutations could in principle cause tumors via any of several Ras effector pathways. In addition, the diverse types of tumors observed in individuals with neurofibromatosis (for review, see Cichowski and Jacks, 2001) could result from hyperactivation of distinct Ras effector pathways. The Raf pathway has been viewed previously as a more relevant effector pathway than the PI3K pathway, mostly because the importance of Ras in the activation of PI3K under physiological conditions remains controversial. In particular, although it is clear that the oncogenic *Ras*$^{V12}$ mutant is sufficient to activate PI3K (Rodriguez-Viciana et al., 1994), it has sometimes been difficult to demonstrate that wild-type Ras is necessary for PI3K activation (Prober and Edgar, 2002). Presumably, this difficulty reflects the fact that PI3K can be activated by Ras-independent as well as Ras-dependent mechanisms, such as direct activation by activated receptor tyrosine kinases or by PIKE-L (phosphatidylinositol kinase enhancer) (Escobedo et al., 1991; Rong et al., 2004). However, more recently, it has been demonstrated that PI3K and Akt are hyperactivated in several *Nf1* mutant cell types and that this hyperactivation is Ras dependent (Dasgupta et al., 2005; Johannessen et al., 2005). Furthermore, PI3K activation plays an essential functional role in *Nf1*$^{-}$-mediated growth defects, as was demonstrated by the observation that PI3K- and Akt-dependent Tor activation was necessary for the proliferation defects of *Nf1* mutants to occur: application of rapamycin, a Tor inhibitor, attenuated the ability of *Nf1* mutant cells to proliferate (Johannessen et al., 2005). These observations demonstrate that PI3K and Akt play key roles in at least some aspects of *Nf1*$^{-}$-induced tumor growth.

Our results are consistent with these observations. By comparing the effects on perineurial glial growth of peripheral–glial expression of activated *Raf*, *PI3K*, or *Ral*, we were able to demonstrate that activation of PI3K, not Raf or Ral, was sufficient to promote perineurial glial growth and that PI3K activity in the peripheral glia was necessary to observe the nonautonomous effect of activated Ras on perineurial glial growth. We similarly showed that Akt activity was necessary and sufficient to mediate the growth-promoting effects of PI3K. However, whereas Dasgupta et al. (2005) and Johannessen et al. (2005) observed that Tor activation was critical for the PI3K- and Akt-dependent growth regulation of *Nf1* mutant cells, we observed a critical role for the PI3K- and Akt-dependent inhibition of the transcription factor FOXO. It is possible that the phenotype observed by Dasgupta et al. (2005) and Johannessen et al. (2005) reflects the well characterized ability of PI3K–Tor to activate growth cell autonomously (Hay and Sonenberg, 2004), whereas the phenotype we report reflects nonautonomous growth regulation. In this view, PI3K and Akt regulate autonomous and nonautonomous growth via the Tor and FOXO pathways, respectively.

FOXO presumably inhibits the growth-promoting effects of PI3K and Akt by transcriptional regulation of target genes. Candidate FOXO target genes include those encoding the molecules oversecreted by *Nf1*$^{-}$ Schwann cells (Yang et al., 2003), whereas other targets might be represented in the distinct transcript profiles exhibited by *Nf1*$^{-}$ Schwann cells (Mashour et al., 2001) or malignant peripheral nerve sheath tumors (Miller et al., 2006) compared with wild-type Schwann cells. For example, Schwann cells from neurofibromas, but not normal Schwann cells, express the epidermal growth factor (EGF) receptor (DeClue et al., 2000). Other potential targets include genes encoding growth factors, although ectopic expression within the peripheral glia of two candidate genes, *Hedgehog* and the EGF ligands *spitz* and *gurken*, failed to induce perineurial glial growth (Lavery and Stern, unpublished observation). Additional experiments will be required to identify the FOXO targets that regulate nonautonomous growth in peripheral nerves.

## References


Auld VJ, Fetter RD, Broadie K, Goodman CS (1995) Gliotactin, a novel transmembrane protein on peripheral glia, is required to form the blood-nerve barrier in *Drosophila*. Cell 81:757–767.

Bourne HR, Sanders DA, McCormick F (1991) The GTPase superfamily: conserved structure and molecular mechanism. Nature 349:117–127.

Brand A, Perrimon N (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118:401–415.

Brand AH, Perrimon N (1994) Raf acts downstream of the EGF receptor to determine dorsoventral polarity during *Drosophila* oogenesis. Genes Dev 8:629–639.

Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J, Greenberg ME (1999) Akt promotes cell survival by phosphorylating and inhibiting a forkhead transcription factor. Cell 96:857–868.

Cichowski K, Jacks T (2001) NF1 tumor suppressor gene function: narrowing the GAP. Cell 104:593–604.

Colomar A, Robitaille R (2004) Glial modulation of synaptic transmission at the neuromuscular junction. Glia 47:284–289.

Dasgupta B, Yi Y, Chen DY, Weber JD, Gutmann DH (2005) Proteomic analysis reveals hyperactivation of the mammalian target of rapamycin pathway in neurofibromatosis 1-associated human and mouse brain tumors. Cancer Res 65:2755–2760.

DeClue JE, Heffelfinger S, Benvenuto G, Ling B, Li S, Rui W, Vass WC, Viskochil D, Ratner N (2000) Epidermal growth factor receptor expression in neurofibromatosis type 1-related tumors and NF1 animal models. J Clin Invest 105:1233–1241.

Edwards JS, Swales LS, Bate M (1993) The differentiation between neuroglia and connective tissue sheath in insect ganglia revisited: the neural lamella and perineurial sheath cells are absent in a mesodermless mutant of *Drosophila*. J comp Neurol 333:301–308.

Escobedo JA, Navankasattusas S, Kavanaugh WM, Milfay D, Fried VA, Williams LT (1991) cDNA cloning of a novel 85 kd protein that has SH2 domains and regulates binding of PI3-kinase to the PDGF beta-receptor. Cell 65:75–82.

Feany MB, Quinn WG (1995) A neuropeptide gene defined by the *Drosophila* memory mutant *amnesiac.* Science 268:869–873.

Franke TF, Yang SI, Chan TO, Datta K, Kazlauskas A, Morrison DK, Kaplan DR, Tsichlis PN (1995) The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. Cell 81:727–736.

Halfar K, Rommel C, Stocker H, Hafen E (2001) Ras controls growth, survival and differentiation in the *Drosophila* eye by different thresholds of MAP kinase activity. Development 128:1687–1696.

Hay N, Sonenberg N (2004) Upstream and downstream of mTOR. Genes Dev 18:1926–1945.

Hofer F, Fields S, Schneider C, Martin GS (1994) Activated Ras interacts with the Ral guanine nucleotide dissociation stimulator. Proc Natl Acad Sci USA 91:11089–11093.

Hwangbo DS, Gersham B, Tu MP, Palmer M, Tatar M (2004) *Drosophila* dFOXO controls lifespan and regulates insulin signalling in brain and fat body. Nature 429:562–566.

Ito K, Urban J, Technau GM (1995) Distribution, classification, and development of *Drosophila* glial cells in late embryonic ventral nerve cord. Roux's Arch Dev Biol 204:284–307.

Johannessen CM, Reczek EE, James MF, Brems H, Legius E, CichowskI K (2005) The NF1 tumor suppressor critically regulates TSC2 and mTOR. Proc Natl Acad Sci USA 102:8573–8578.

Junger MA, Rintelen F, Stocker H, Wasserman JD, Vegh M, Radimerski T, Greenberg ME, Hafen E (2003) The *Drosophila* forkhead transcription factor FOXO mediates the reduction in cell number associated with reduced insulin signaling. J Biol 2:20.

Karim FD, Rubin GM (1998) Ectopic expression of activated Ras1 induces hyperplastic growth and increased cell death in *Drosophila* imaginal tissues. Development 125:1–9.

Kluwe L, Friedrich R, Mautner VF (1999) Loss of NF1 allele in Schwann cells but not in fibroblasts derived from an NF1-associated neurofibroma. Genes Chromosomes Cancer 24:283–285.

Kolch W, Heidecker G, Lloyd P, Rapp UR (1991) Raf-1 protein kinase is required for growth of induced NIH/3T3 cells. Nature 349:426–428.

Lee T, Feig L, Montell DJ (1996) Two distinct roles for Ras in a developmentally regulated cell migration. Development 122:409–418.

Leevers SJ, Weinkove D, MacDougall LK, Hafen E, Waterfield MD (1996) The *Drosophila* phosphoinositide 3-kinase Dp110 promotes cell growth. EMBO J 15:6584–6594.

Leiserson WM, Harkins EW, Keshishian H (2000) Fray, a *Drosophila* serine/threonine kinase homologous to mammalian PASK, is required for axonal ensheathment. Neuron 28:793–806.

Mashour GA, Ratner N, Khan GA, Wang HL, Martuza RL, Kurtz A (2001) The angiogenic factor midkine is aberrantly expressed in NF1-deficient Schwann cells and is a mitogen for neurofibroma-derived cells. Oncogene 20:97–105.

Miller SJ, Rangwala F, Williams J, Ackerman P, Kong S, Jegga AG, Kaiser S, Aronow BJ, Frahm S, Kluwe L, Mautner V, Upadhyaya M, Muir D, Wallace M, Hagen J, Quelle DE, Watson MA, Perry A, Gutmann DH, Ratner N (2006) Large-scale molecular comparison of human schwann cells to malignant peripheral nerve sheath tumor cell lines and tissues. Cancer Res 66:2584–2591.

Parmantier E, Lynn B, Lawson D, Turmaine M, Namini SS, Chakrabarti L, McMahon AP, Jessen KR, Mirsky R (1999) Schwann cell-derived Desert hedgehog controls the development of peripheral nerve sheaths. Neuron 23:713–724.

Perrimon N, Lanjuin A, Arnold C, Noll E (1996) Zygotic lethal mutations with maternal effect phenotypes in *Drosophila melanogaster*. II. Loci on the second and third chromosomes identified by P-element-induced mutations. Genetics 144:1681–1692.

Prober DA, Edgar BA (2002) Interactions between Ras1, dMyc, and dPI3K signaling in the developing *Drosophila* wing. Genes Dev 16:2286–2299.

Puig O, Marr MT, Ruhf ML, Tjian R (2003) Control of cell number by *Drosophila* FOXO: downstream and feedback regulation of the insulin receptor pathway. Genes Dev 17:2006–2020.

Rodriguez-Viciana P, Warne PH, Dhand R, Vanhaesebroeck B, Gout I, Fry MJ, Waterfield MD, Downward J (1994) Phosphatidylinositol-3-OH kinase as a direct target of Ras. Nature 370:527–532.

Rong R, Tang X, Gutmann DH, Ye K (2004) Neurofibromatosis 2 (NF2) tumor suppressor merlin inhibits phosphatidylinositol 3-kinase through binding to PIKE-L. Proc Natl Acad Sci USA 101:18200–18205.

Sawamoto K, Winge P, Koyama S, Hirota Y, Yamada C, Miyao S, Yoshikawa S, Jin MH, Kikuchi A, Okano H (1999) The *Drosophila* Ral GTPase regulates developmental cell shape changes through the Jun $NH_2$-terminal kinase pathway. J Cell Biol 146:361–372.

Scheid MP, Woodgett JR (2001) PKB/AKT: functional insights from genetic models. Nat Rev Mol Cell Biol 2:760–768.

Sepp KJ, Auld VJ (1999) Conversion of lacZ enhancer trap lines to GAL4 lines using targeted transposition in *Drosophila melanogaster*. Genetics 151:1093–1101.

Sepp KJ, Schulte J, Auld VJ (2000) Developmental dynamics of peripheral glia in *Drosophila melanogaster.* Glia 30:122–135.

Sepp KJ, Schulte J, Auld VJ (2001) Peripheral glia direct axon guidance across the CNS/PNS transition zone. Dev Biol 238:47–63.

Serra E, Rosenbaum T, Winner U, Aledo R, Ars E, Estivill X, Lenard HG, Lazaro C (2000) Schwann cells harbor the somatic NF1 mutation in neurofibromas: evidence of two different Schwann cell subpopulations. Hum Mol Genet 9:3055–3064.

Sherman LS, Atit R, Rosenbaum T, Cox AD, Ratner N (2000) Single cell Ras-GTP analysis reveals altered Ras activity in a subpopulation of neurofibroma Schwann cells but not fibroblasts. J Biol Chem 275:30740–30745.

Shiga Y, Tanaka-Matakatsu M, Hayashi S (1996) A nuclear GFP/ beta-galactosidase fusion protein as a marker for morphogenesis in living *Drosophila*. Dev Growth Differ 38:99–106.

Soehnge H, Huang X, Becker M, Whitley P, Conover D, Stern M (1996) A neurotransmitter transporter encoded by the *Drosophila* inebriated gene, Proc Natl Acad Sci USA 93:13262–13267.

Yager J, Richards S, Hekmat-Scafe DS, Hurd DD, Sundaresan V, Caprette DR, Saxton WM, Carlson JR, Stern M (2001) Control of *Drosophila* perineurial glial growth by interacting neurotransmitter-mediated signaling pathways. Proc Natl Acad Sci USA 98:10445–10450.

Yang FC, Ingram DA, Chen S, Hingtgen DM, Ratner N, Monk KR, Clegg T, White H, Mead L, Wenning MJ, Williams DA, Kapur R, Atkinson SJ, Clapp DW (2003) Neurofibromin-deficient Schwann cells secrete a potent migratory stimulus for Nf1+/− mast cells. J Clin Invest 112:1851–1861.

Yuan LL, Ganetzky B (1999) A glial-neuronal signaling pathway revealed by mutations in a neurexin-related protein. Science 283:1343–1345.

Zhu Y, Ghosh P, Charnay P, Burns DK, Parada LF (2002) Neurofibromas in NF1: Schwann cell origin and role of tumor environment. Science 296:920–922.

PLoS GENETICS

# A PI3-Kinase–Mediated Negative Feedback Regulates Neuronal Excitability

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## Abstract

Use-dependent downregulation of neuronal activity (negative feedback) can act as a homeostatic mechanism to maintain neuronal activity at a particular specified value. Disruption of this negative feedback might lead to neurological pathologies, such as epilepsy, but the precise mechanisms by which this feedback can occur remain incompletely understood. At one glutamatergic synapse, the Drosophila neuromuscular junction, a mutation in the group II metabotropic glutamate receptor gene (*DmGluRA*) increased motor neuron excitability by disrupting an autocrine, glutamate-mediated negative feedback. We show that *DmGluRA* mutations increase neuronal excitability by preventing PI3 kinase (PI3K) activation and consequently hyperactivating the transcription factor Foxo. Furthermore, glutamate application increases levels of phospho-Akt, a product of PI3K signaling, within motor nerve terminals in a *DmGluRA*-dependent manner. Finally, we show that PI3K increases both axon diameter and synapse number via the Tor/S6 kinase pathway, but not Foxo. In humans, PI3K and group II mGluRs are implicated in epilepsy, neurofibromatosis, autism, schizophrenia, and other neurological disorders; however, neither the link between group II mGluRs and PI3K, nor the role of PI3K-dependent regulation of Foxo in the control of neuronal excitability, had been previously reported. Our work suggests that some of the deficits in these neurological disorders might result from disruption of glutamate-mediated homeostasis of neuronal excitability.

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## Introduction

Negative feedback processes, which can enable maintenance of neuronal homeostasis, are widely observed in neuronal systems [1–3]. For example, neuronal silencing via tetrodotoxin application both *in vivo* and *in vitro* increases excitability [4–6]. This effect occurs *in vitro* via both increased sodium currents and decreased potassium currents. However, the signaling pathways responsible for these excitability changes remain unclear.

The mammalian group II metabotropic glutamate receptors, which are G-protein coupled receptors activated by glutamate, are well positioned to mediate negative feedback. When localized presynaptically, these receptors can act as autoinhibitors of glutamate release [7–10]. Because these receptors are located outside of the active zone [11], activation is thought to occur only during conditions of elevated glutamate release and might serve to prevent glutamate-mediated neurotoxicity. Agonists for these receptors are proposed for treatment of schizophrenia, anxiety and epilepsy, among others [12,13], but the mGluR-dependent signaling pathways that underlie these disorders remain unidentified. Furthermore, although many of the acute effects of group II mGluR activation on neuronal physiology have been elucidated [14,15], possible long term effects on neuronal function, such as through changes in ion channel gene expression, remain essentially unexplored.

In Drosophila, the single *DmGluRA* gene encodes a protein most similar to the mammalian group II mGluR [16]. DmGluRA is located presynaptically at the neuromuscular junction (nmj), which suggests that DmGluRA might regulate transmitter release from motor neurons. Elimination of *DmGluRA* by the null mutation *DmGluRA*$^{112b}$, or by RNAi-mediated *DmGluRA* knockdown specifically in motor neurons, increases neuronal excitability [16]. Given that glutamate is the excitatory neurotransmitter from Drosophila motor neurons, the increased excitability of *DmGluRA* mutants raised the possibility that DmGluRA decreases motor neuron excitability upon activation by glutamate released from motor nerve terminals. In this view, DmGluRA would mediate an activity-dependent negative feedback on excitability. However, the mechanism by which this negative feedback is accomplished was not elucidated.

Here we show that glutamate-mediated activation of DmGluRA decreases neuronal excitability by activating the lipid kinase PI3 kinase (PI3K), which promotes growth and inhibits apoptosis in various cell types. In particular, we report that transgene-induced inhibition of PI3K in motor neurons confers neuronal excitability phenotypes similar to *DmGluRA*$^{112b}$, whereas transgene-induced activation of PI3K confers the opposite excitability phenotypes. We also show that PI3K activation in motor neurons suppresses the increased excitability of *DmGluRA*$^{112b}$, and glutamate application to motor nerve terminals activates PI3K in a DmGluRA-dependent manner. Finally, we show that altered PI3K activity regulates both axon diameter and synapse number, and that these effects on neuronal growth are mediated by the Tor/S6 kinase pathway, whereas the effects of PI3K on neuronal excitability are mediated by the transcription factor Foxo. We conclude that negative feedback of Drosophila motor neuron excitability occurs

## Author Summary

Use-dependent downregulation of neuronal excitability (negative feedback) can act to maintain neuronal activity within specified levels. Disruption of this homeostasis can lead to neurological disorders, such as epilepsy. Here, we report a novel mechanism for negative feedback control of excitability in the Drosophila larval motor neuron. In this mechanism, activation by the excitatory neurotransmitter glutamate of metabotropic glutamate receptors (mGluRs) located at motor nerve terminals decreases excitability by activating PI3 kinase (PI3K), consequently causing the phosphorylation and inhibition of the transcription factor Foxo. Foxo inhibition, in turn, decreases neuronal excitability. These observations are of interest for two reasons. First, our observation that PI3K activity regulates neuronal excitability is of interest because altered PI3K activity is implicated in a number of neurological disorders, such as autism and neurofibromatosis. Our results raise the possibility that altered excitability might contribute to the deficits in these disorders. Second, our observation that group II metabotropic glutamate receptors (mGluRs) activate PI3K is of interest because group II mGluRs are implicated in epilepsy, anxiety disorders, and schizophrenia. Yet the downstream signaling pathways affected by these treatments are incompletely understood. Our results raise the possibility that the PI3K pathway might be an essential mediator of signalling by these mGluRs.


via the glutamate-induced activation of DmGluRA autoreceptors, causing the PI3K-dependent inhibition of Foxo and a consequent decrease in neuronal excitability. A similar negative feedback operating in the mammalian CNS might underlie neuronal disorders involving the group II mGluRs or PI3K.

## Results

### Drosophila mGluRA (DmGluRA) Affects the Rate of Onset of Long-Term Facilitation (LTF), a Reporter for Motor Neuron Excitability

The increase in neuronal excitability conferred by the *DmGluRA*$^{112b}$ null mutation is manifested by an increased rate of onset of a form of synaptic plasticity termed long-term facilitation (LTF) [16,17], which is induced when a motor neuron is subjected to repetitive nerve stimulation at low bath [$Ca^{2+}$]. At a certain point in the stimulus train, an abrupt increase in transmitter release and hence muscle depolarization (termed excitatory junctional potential, or ejp) is observed (Figure 1A). LTF not only increases ejp amplitude, but also ejp duration, indicative of prolonged and asynchronous transmitter release (Figure 1A). This abrupt increase in the amount and duration of transmitter release is caused by an abrupt increase in the duration of nerve terminal depolarization and hence $Ca^{2+}$ influx, and reflects a progressive increase in motor neuron excitability induced by the repetitive nerve stimulation: when an excitability threshold is reached, LTF occurs [17–19].

In Drosophila, many genotypes that increase motor neuron excitability by decreasing $K^+$ currents or increasing $Na^+$ currents increase the rate of onset of LTF. For example, altered activities of *frequenin* and *Hyperkinetic*, which act via $K^+$ channels, or *paralytic* and *pumilio*, which act via $Na^+$ channels, each increase the rate of onset of LTF [18–24]. By increasing motor neuron excitability, the genotypes described above apparently bring excitability closer to the threshold required to evoke LTF and consequently decrease the number of prior nerve stimulations required to reach this threshold. In these genotypes, the prolonged nerve terminal depolarizations that triggered LTF were revealed by recording ejps and simultaneously recording extracellularly electrical activity within the peripheral nerves during LTF onset. It was found that LTF onset was accompanied by the appearance within peripheral nerves of supernumerary action potentials occurring at about 10 msec intervals following the initial induced action potential [18–25]. Several lines of evidence suggested that these supernumerary action potentials arose in motor axons and were responsible for the increased transmitter release underlying LTF. First, the number of these supernumerary action potentials correlated with ejp duration, and second, these supernumerary action potentials often preceded depolarizing steps in the asynchronous, multi-step ejps that occurred after LTF onset. Similar supernumerary action potentials were observed following nerve stimulation in the *eag Sh* double mutant, in which two distinct K channel $\alpha$ subunits are simultaneously eliminated, and which consequently exhibits extreme neuronal hyperexcitability. In the *eag Sh* double mutant, these supernumerary action potentials arise in the motor nerve terminals and exhibit retrograde propagation [25]. It was suggested that the supernumerary action potentials were caused by, and also prolonged, motor nerve terminal depolarization, and thus participated in a positive feedback loop prolonging depolarization [25]. This positive feedback loop presumably underlies the abrupt, threshold-like onset of LTF.

The observation that *mGluR*$^{112b}$ increases the rate of onset of LTF suggested that *DmGluRA*$^{112b}$ increases motor neuron excitability as well [16]. To confirm this suggestion, we simultaneously recorded peripheral nerve electrical activity and ejps during LTF induced by 10 Hz stimulus trains. As previously observed in the hyperexcitable genotypes described above [18–25], we found that the abrupt onset of LTF in *mGluRA*$^{112b}$ was accompanied in the nerve by the appearance of supernumerary action potentials (Figure S1). This observation confirmed that LTF onset in *mGluRA*$^{112b}$ was caused by prolonged motor nerve terminal depolarization, and hence that *mGluRA*$^{112b}$ increases neuronal excitability. Thus, as suggested previously [16], it appears that DmGluRA mediates an activity-dependent inhibition of neuronal excitability. In this view, glutamate release from motor nerve terminals downregulates subsequent neuronal activity by activating presynaptic DmGluRA autoreceptors, which then decrease excitability. Elimination of DmGluRA disrupts this negative feedback and prevents the decrease in excitability from occurring.

### The *DmGluRA*$^{112b}$-Null Mutation Increases Neuronal Excitability by Preventing PI3K Activation

In addition to increasing neuronal excitability, *DmGluRA*$^{112b}$ also decreases arborization and synapse number at the larval neuromuscular junction [16]. This phenotype is also observed in larval motor neurons with decreased activity of PI3K [26]. This observation raised the possibility that DmGluRA might exert its effects on neuronal excitability as well as synapse formation via PI3K activity. To test the possibility that PI3K mediates the effects of DmGluRA on neuronal excitability, we used the *D42 Gal4* driver [27,28] to overexpress transgenes expected to alter activity of the motor neuron PI3K pathway. We found that inhibiting the PI3K pathway by motor neuron-specific overexpression of either the phosphatase *PTEN*, which opposes the effect of PI3K, or the dominant-negative *PI3K*$^{DN}$ [29], each significantly increased the rate of onset of LTF, similarly to that of *DmGluRA*$^{112b}$ (Figure 1A and 1B). In contrast, we found that activating the PI3K pathway by expression of the constitutively active *PI3K-CAAX* [29], or via

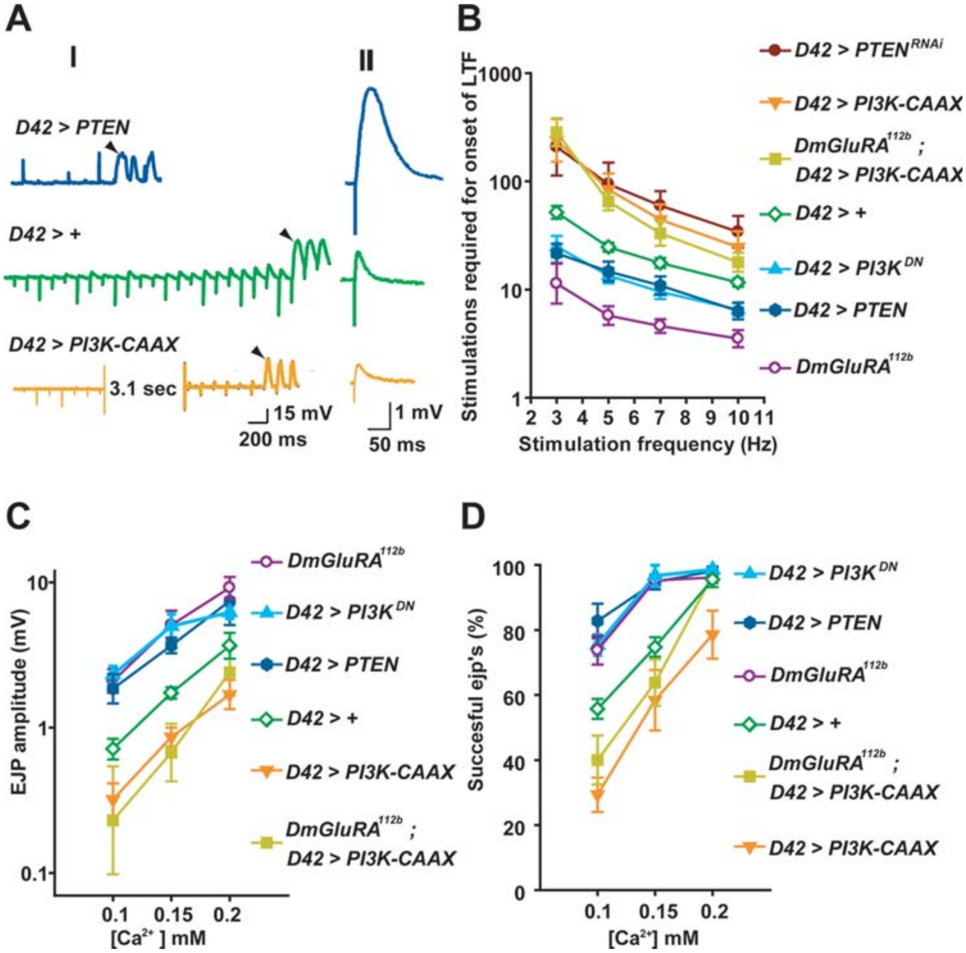


**Figure 1. DmGluRA activity inhibits neuronal excitability via activation of the PI3K pathway.** The motor neuron GAL4 driver *D42* was used to drive expression of all transgenes. For all LTF experiments, the bath solution contained 0.15 mM [$Ca^{2+}$] and 100 μM quinidine, which is a $K^+$ channel blocker that sensitizes the motor neuron and enables LTF to occur and measured even in hypoexcitable neurons. A) Representative traces showing the decreased rate of onset of long-term facilitation (LTF) (I) and decreased excitatory junction potential (ejp) amplitude (II) in larvae overexpressing *PI3K-CAAX* in motor neurons compared to wildtype at the indicated [$Ca^{2+}$], and the increased rate of onset of LTF and ejp amplitude in larvae overexpressing *PTEN*. Arrowheads indicate the increased and asynchronous ejps, indicative of onset of LTF. In (II), ejps are averages of 180 responses for each genotype. B) Number of stimulations required to induce LTF (Y axis) at the indicated stimulus frequencies (X axis) in the indicated genotypes. Geometric means+/−SEMs are shown. From top to bottom, $n = 6$, 12, 7, 18, 12, 21, and 6 respectively, for each genotype. One-way ANOVA and Fisher's LSD gave the following differences, at 10 Hz, 7 Hz, 5 Hz and 3 Hz, respectively: For *D42*>+: vs. *D42*>*PI3K-CAAX*, p = 0.013, 0.0021, 0.0002, <0.0001; vs. *D42*>PTEN, p = 0.011, 0.056, 0.079, 0.0054; vs. *D42*>$PTEN^{RNAi}$, p = 0.0018, 0.0004, 0.0006, 0.0014; vs. *D42*>$PI3K^{DN}$, p = 0.035, 0.036, 0.05, 0.038; vs. $mGluR^{112b}$, p = 0.0012, 0.0005, 0.0004, 0.0009. For $mGluR^{112b}$, *D42*>*PI3K-CAAX* vs: $mGluR^{112b}$, p = 0.0003, <0.0001, <0.0001, <0.0001; vs. *D42*>*PI3K-CAAX*, p = 0.33, 0.34. 0.46, 0.62. C) Mean ejp amplitudes (Y axis) at the indicated [$Ca^{2+}$] (X axis), from the indicated genotypes. Larval nerves were stimulated at a frequency of 1 Hz, and 10 responses were measured from each of nine larvae (for *D42*>*PI3K-CAAX* and *D42*>$PI3K^{DN}$) and for six larvae from other genotypes. Means+/−SEMs are shown. One-way ANOVA and Fisher's LSD gave the following differences, at 0.1 mM, 0.15 mM and 0.2 mM [$Ca^{2+}$], respectively: For *D42*>+: vs. *D42*>*PI3K-CAAX*, p = 0.028, 0.05, 0.04; vs. *D42*>PTEN, p = 0.017, 0.03, 0.06; vs. *D42*>$PI3K^{DN}$, p = 0.0018, 0.0033, 0.14; vs. $mGluR^{112b}$, p = 0.0077, 0.0029, 0.01. For $mGluR^{112b}$, *D42*>*PI3K-CAAX* vs: $mGluR^{112b}$, p<0.0001, <0.0001, 0.0001; vs. *D42*>*PI3K-CAAX*, p = 0.70, 0.47, 0.26. D) Effects of altered PI3K pathway activity on failures of transmitter release. Mean transmitter release success rate +/−SEMs (Y axis) at the indicated $Ca^{2+}$ concentration (X axis) for the indicated genotypes. Larval nerves were stimulated at 1 Hz. 10 responses were collected per nerve from each of 6 larvae for the given genotype and at the given $Ca^{2+}$ concentration. One-way ANOVA and Fisher's LSD gave the following differences, at 0.1 mM, 0.15 mM and 0.2 mM [$Ca^{2+}$], respectively: For *D42*>+: vs. *D42*>*PI3K-CAAX*, p = 0.0023, 0.023, 0.001; vs. *D42*>PTEN, p = 0.0014, 0.0068, 0.69; vs. *D42*>$PI3K^{DN}$, p = 0.011, 0.003, 0.63; vs. $mGluR^{112b}$, p = 0.027, 0.0053, 0.99. For $mGluR^{112b}$, *D42*>*PI3K-CAAX* vs: $mGluR^{112b}$, p = 0.0001, <0.0001, 0.94; vs. *D42*>*PI3K-CAAX*, p = 0.21, 0.45, 0.0008.
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RNAi-mediated inhibition of *PTEN*, decreased rate of onset of LTF (Figure 1A and 1B). As was described above for $mGluRA^{112b}$, LTF onset was accompanied by the appearance of supernumerary action potentials in the nerve (Figure S1) demonstrating that altered excitability is responsible for the altered rate of onset of LTF in these genotypes.

The rate of LTF onset described above was measured in the presence of the potassium channel blocking drug quinidine, which moderately increases neuronal excitability and hence rate of onset of LTF in the larval motor neuron. Quinidine application sensitizes the motor neuron to the effects of the nerve stimulation and enables LTF to occur reliably in genotypes with low excitability, even at lower stimulus frequencies. To demonstrate that altered PI3K activity does not alter rate of onset of LTF by altering sensitivity to quinidine, we compared the timing of LTF onset in the absence of quinidine in wildtype larvae and in larvae with inhibited PI3K. We found that inhibiting PI3K activity in motor neurons significantly accelerated LTF onset even in the absence of quinidine (Figure S2) demonstrating that altered sensitivity of motor neurons to quinidine does not underlie the altered onset rate of LTF that we observe.

In addition to effects on LTF, mutations that alter motor neuron excitability can alter basal transmitter release and hence ejp amplitude at low bath $Ca^{2+}$ concentrations, at which $Ca^{2+}$ influx would be limiting for vesicle fusion to occur. For example, mutations in *ether-a go-go (eag)*, which encodes a potassium channel $\alpha$ subunit, increase transmitter release about two-fold [25], whereas a mutation in the sodium channel gene *paralytic* decreases transmitter release by increasing the frequency at which nerve stimulation failed to evoke any vesicle fusion, termed "failure" of vesicle release [30]. Presumably altered excitability affects the amplitude or duration of the action potential and consequently the amount of $Ca^{2+}$ influx through voltage-gated channels. We found that *DmGluRA*$^{112b}$ also increased ejp amplitude and hence basal transmitter release at three low bath $Ca^{2+}$ concentrations tested (Figure 1C), which is consistent with increased motor neuron excitability in this genotype. We found that decreasing PI3K pathway activity via motor neuron overexpression of *PI3K*$^{DN}$ or *PTEN* also increased transmitter release to levels similar to *DmGluRA*$^{112b}$, whereas increasing PI3K pathway activity via overexpression of *PI3K-CAAX* decreased basal transmitter release (Figure 1C).

The *DmGluRA*$^{112b}$ mutation also decreased the frequency at which failures of vesicle release occur, particularly at the lower $Ca^{2+}$ concentrations tested (Figure 1D). This observation confirms that the effect of *DmGluRA*$^{112b}$ on ejp amplitude is presynaptic. We also observed a decreased frequency of failures when the PI3K pathway was inhibited by motor neuron expression of *PI3K*$^{DN}$ or *PTEN* (Figure 1D). In contrast, motor neuron overexpression of *PI3K-CAAX* increased the frequency of failures (Figure 1D). Therefore, with three electrophysiological readouts, the *DmGluRA*$^{112b}$ mutant phenotype was mimicked by decreased activity of the PI3K pathway, whereas increasing PI3K pathway activity conferred opposite effects.

These observations support the notion that loss of DmGluRA increases motor neuron excitability by preventing the activation of PI3K. If so, then motor neuron expression of *PI3K-CAAX*, which will be active independently of DmGluRA, is predicted to suppress the *DmGluRA*$^{112b}$ hyperexcitability. To test this possibility, we drove motor-neuron expression of *PI3K-CAAX* in a *DmGluRA*$^{112b}$ background and found a rate of onset of LTF and ejp amplitude that was very similar to what was observed when *PI3K-CAAX* was expressed in a wildtype background, but significantly different from *DmGluRA*$^{112b}$ (Figure 1B, Figure 1C). In addition, motor neuron-specific expression of *PI3K-CAAX* increased failure frequency at the two lower [$Ca^{2+}$] tested to the same level in *DmGluRA*$^{112b}$ larvae as in wildtype (Figure 1D). We conclude that hyperexcitability of the *DmGluRA*$^{112b}$ mutant results from inability to activate PI3K.

## Glutamate Application Increases Levels of Phosphorylated Akt in Motor Nerve Terminals in a DmGluRA-Dependent Fashion

The results described above suggest that glutamate release from motor nerve terminals as a consequence of motor neuron activity activates PI3K within motor nerve terminals via DmGluRA autoreceptors. To test this possibility directly, we measured the ability of glutamate applied to the neuromuscular junction to activate PI3K within motor nerve terminals. To assay for PI3K activity we applied an antibody specific for the phosphorylated form of the kinase Akt (p-Akt), which is increased by elevated PI3K pathway activity. The usefulness of this antibody for specific detection of Drosophila p-Akt has been previously demonstrated [31–33]. The ability to detect p-Akt in larval motor nerve terminals overexpressing *PI3K-CAAX*, but not in wildtype (Figure 2), further validates this antibody as a PI3K reporter.

We compared p-Akt levels in wildtype versus *DmGluRA*$^{112b}$ motor nerve terminals immediately prior to or following a 1 minute application of 100 $\mu$M glutamate. We found that glutamate application strongly increased p-Akt levels in wildtype larvae, but not in the *DmGluRA*$^{112b}$ larvae (Figure 2), demonstrating that glutamate application increases nerve terminal p-Akt levels, and that DmGluRA activity is required for this increase.

We found that DmGluRA activity was required presynaptically for this p-Akt increase: motor neuron-specific expression of a *DmGluRA* RNAi construct [16], blocked the ability of glutamate to increase p-Akt levels (Figure 2). In [16] it was reported that expression of *DmGluRA* RNAi decreased, but did not eliminate, mGluRA immunoreactivity, suggesting that this transgene decreases, but does not eliminate, glutamate-mediated signalling via mGluRA. The ability of this transgene to block glutamate-mediated induction of p-Akt suggests that activation of PI3K by glutamate is sensitive to mGluRA levels and requires a minimum level of mGluRA expression. In contrast, expression of the *DmGluRA* RNAi construct in the muscle, with use of the *24B Gal4* driver, did not inhibit p-Akt levels: p-Akt intensity following 1 minute of glutamate application was not significantly different from wildtype (17.6+/−2.9, $p = 0.59$).

To determine if PI3K activity was required presynaptically for this glutamate-induced p-Akt increase, we inhibited PI3K activity by motor neuron-expression of *PI3K*$^{DN}$, and found that this transgene significantly inhibited the ability of glutamate to activate p-Akt (Figure 2). Thus, presynaptic DmGluRA and PI3K activity are both necessary for glutamate to increase p-Akt.

## The Effects of PI3K on Neuronal Excitability Are Mediated by Foxo, not Tor/S6 Kinase

Many effects of the PI3K pathway are mediated by the downstream kinase Akt. Activated Akt phosphorylates targets such as Tsc1/Tsc2, which regulates cell growth via the Tor/S6 Kinase (S6K) pathway [34], Foxo, which regulates apoptosis [35], and GSK3 [36], which mediates at least in part the effects of altered PI3K pathway activity on arborization and synapse number [26]. All of these Akt-mediated phosphorylation events inhibit activity of the target protein.

If PI3K pathway activity decreases neuronal excitability by inhibiting Foxo, then Foxo overexpression is predicted to mimic the hyperexcitability observed when PI3K pathway activity is blocked in motor neurons, whereas loss of Foxo is predicted to mimic the hypoexcitability observed when *PI3K-CAAX* is expressed in motor neurons. To test these predictions, we measured neuronal excitability in larvae carrying the heteroallelic *Foxo*$^{21}$/*Foxo*$^{25}$ null mutant combination [37] and in larvae overexpressing *Foxo*$^{+}$ [38] in motor neurons. We found that overexpression of *Foxo*$^{+}$ increased the rate of onset of LTF, basal transmitter release and frequency of successful ejps to a level very similar to that observed when PI3K pathway activity was decreased (Figure 3) whereas in *Foxo*$^{21}$/*Foxo*$^{25}$ larvae, the rate of onset of LTF, basal transmitter release and frequency of successful ejps were decreased to levels very similar to those observed when *PI3K-CAAX* was expressed in motor neurons (Figure 3). These observations support the notion that PI3K activity decreases excitability by downregulating Foxo activity.

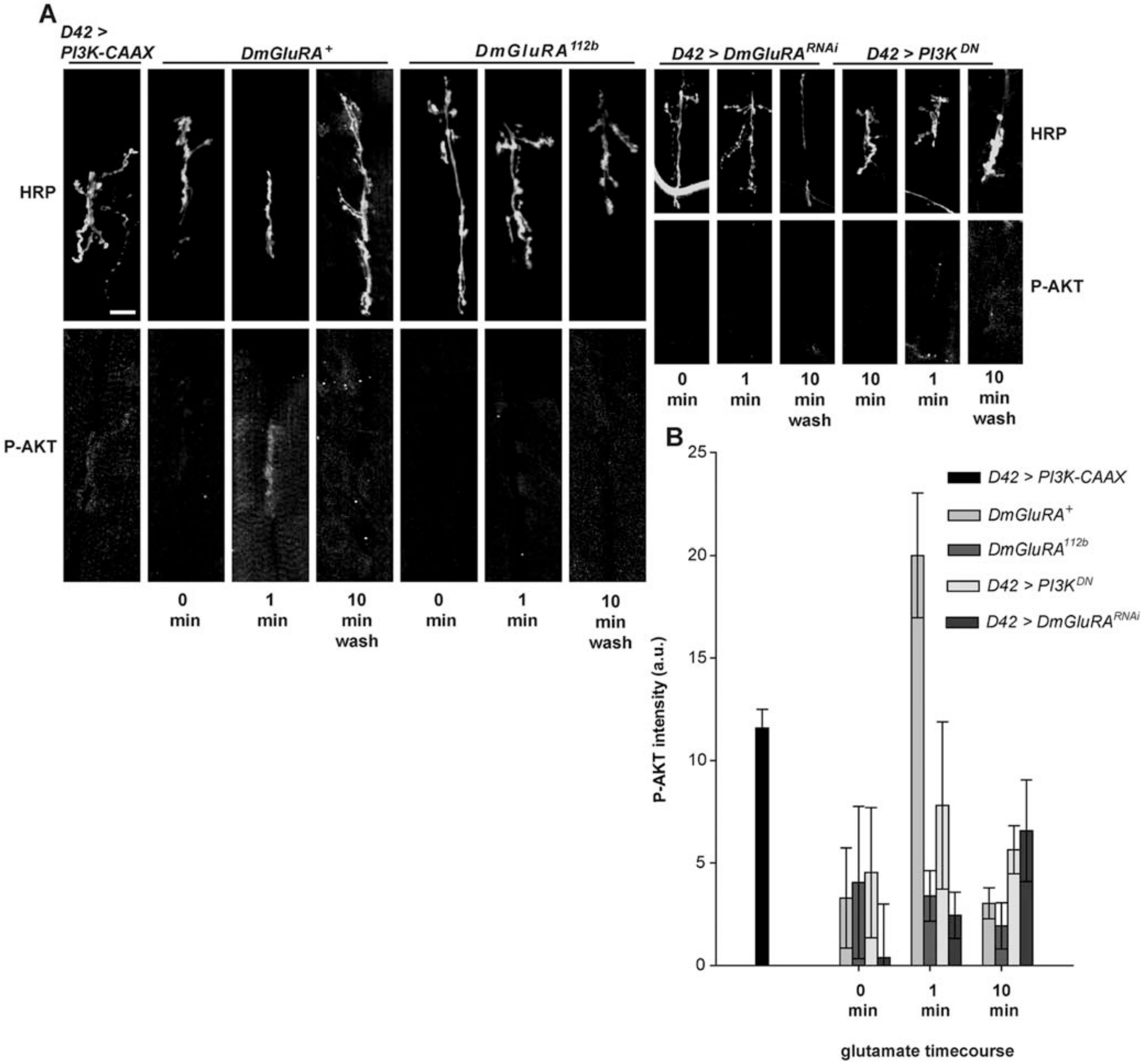


**Figure 2. Glutamate application stimulates presynaptic Akt phosphorylation in *DmGluRA*$^{+}$ but not in *DmGluRA*$^{112b}$ mutant larvae.** A) Representative confocal images of *DmGluRA*$^{+}$, *DmGluRA*$^{112b}$, *D42>DmGluRA*$^{RNAi}$ and *D42>PI3K*$^{DN}$ larvae stained with anti-HRP (upper) and anti-p-Akt (lower) in the indicated conditions. All images are from muscles 7 and 6 of abdominal segment A3 or A4. Scale bar = 20 µm. B) Quantification of phosphorylated Akt (p-Akt) levels in *DmGluRA*$^{+}$, *DmGluRA*$^{112b}$, *D42>DmGluRA*$^{RNAi}$ and *D42>PI3K*$^{DN}$ larvae immediately prior to glutamate application, after 1 min of 100 µM glutamate application (final bath concentration), and 10 min after a wash with glutamate free media. Nerve terminals were outlined with HRP fluorescence as reference. Pixel intensities were quantified using ImageJ software and background subtraction was performed as described in detail in Methods section. Bars represent mean synaptic p-Akt levels +/−SEMs. *D42>PI3K-CAAX* is included as a positive control. One-way ANOVA and Fisher's LSD gave the following significant differences for p-Akt levels one minute after glutamate application. For *DmGluRA*$^{+}$ vs. *DmGluRA*$^{112b}$, $p = 0.0072$; vs. *D42>PI3K*$^{DN}$, $p = 0.0097$; vs. *D42>DmGluRA*$^{RNAi}$, $p < 0.0001$.
doi:10.1371/journal.pgen.1000277.g002

If the hyperexcitability conferred by motor neuron expression of *PI3K*$^{DN}$ results from Foxo hyperactivity, then the *Foxo*$^{21}$/*Foxo*$^{25}$ null combination will suppress this hyperexcitability and confer motor neuron hypoexcitability similar to what is observed in *Foxo*$^{21}$/*Foxo*$^{25}$ larvae in an otherwise wildtype background. We confirmed this prediction: larvae carrying the *Foxo*$^{21}$/*Foxo*$^{25}$ null combination and expressing *PI3K*$^{DN}$ in motor neurons exhibited a rate of onset of LTF, basal transmitter release, and failure frequency very similar to what was observed in the *Foxo*$^{21}$/*Foxo*$^{25}$ null mutant alone (Figure 3), or in larvae expressing *PI3K-CAAX* in motor neurons. We used the *OK6* motor neuron *Gal4* driver rather than *D42* for ease of stock construction in experiments involving *Foxo*$^{21}$/*Foxo*$^{25}$. *OK6* confers motor neuron phenotypes indistinguishable from *D42* in our assays (Figure 3B and not shown).

In addition, if the hypoexcitability conferred by motor neuron expression of *PI3K-CAAX* results from decreased Foxo activity, then co-overexpression of *Foxo*$^{+}$ will suppress this hypoexcitability and confer hyperexcitability similar to what is observed when

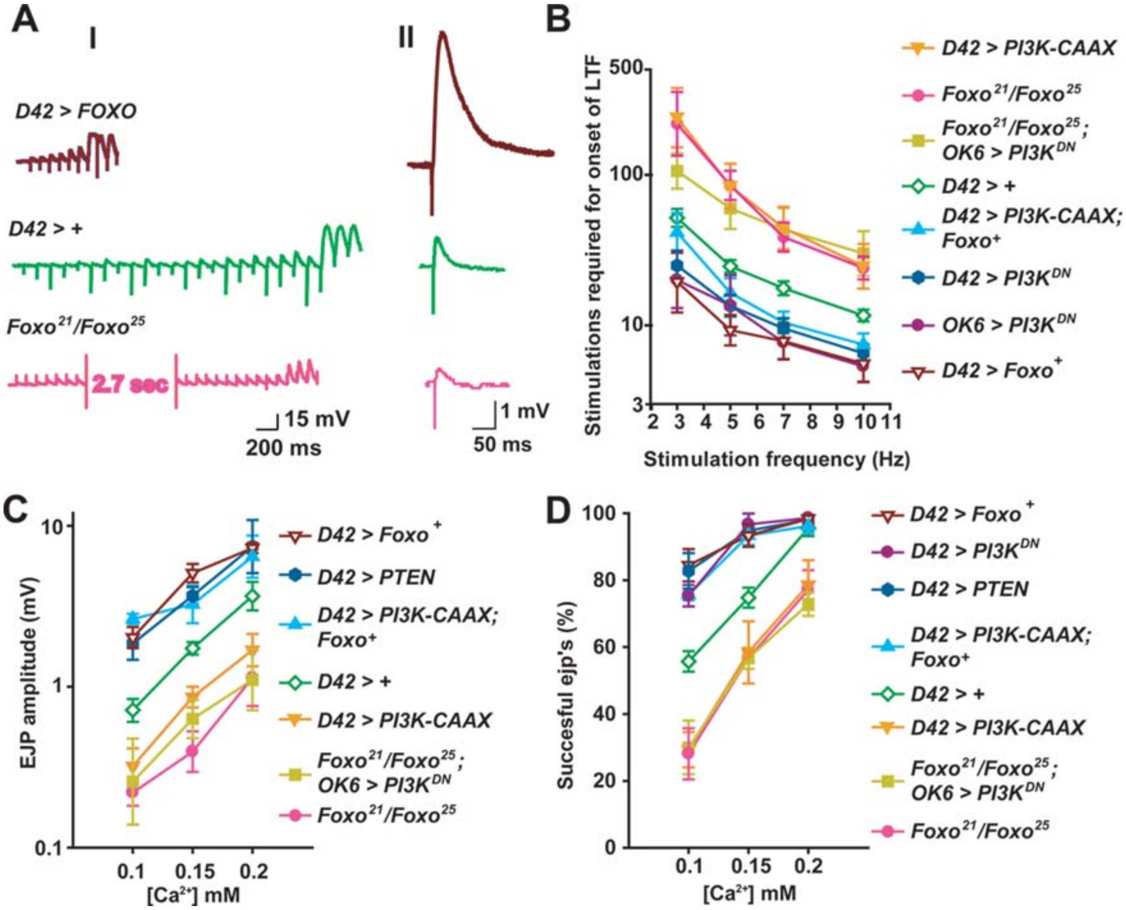


**Figure 3. *Foxo* mediates the effects of *PI3K* on motor neuron excitability.** The *Gal4* driver *D42* was used to drive expression of transgenes in all genotypes except for $Foxo^{21}/Foxo^{25}$; $OK6>PI3K^{DN}$, in which the motor neuron driver *OK6* was used and which behaves similarly to *D42* in this assay. For all LTF experiments, the bath solution contained 0.15 mM [$Ca^{2+}$] and 100 µM quinidine. A) Representative traces showing the decreased rate of onset of LTF (I) and decreased ejp amplitude (II) in $Foxo^{21}/Foxo^{25}$ larvae compared to wildtype at the indicated [$Ca^{2+}$], and the increased rate of onset of LTF and ejp amplitude in larvae overexpressing *Foxo*. Arrowheads indicate the increased and asynchronous ejps, indicative of onset of LTF. In (II), representative traces are averages of multiple ejps. From *top* to *bottom*, $n=23$, 180, and 34 respectively. B) Number of stimulations required to induce LTF (Y axis) at the indicated stimulus frequencies (X axis). Geometric means+/−SEMs are shown. From *top* to *bottom*, $n=12$, 6, 7, 18, 10, 21, 5, and 9 respectively, for each genotype. One-way ANOVA and Fisher's LSD gave the following differences, at 10 Hz, 7 Hz, 5 Hz and 3 Hz, respectively: For *D42*>+ vs. $Foxo^{21}/Foxo^{25}$, p = 0.0096, 0.0069, <0.0001, 0.0007; vs. $D42>Foxo^{+}$, p = 0.0026, 0.0012, <0.0001, 0.0065. For *D42*>*PI3K-CAAX*, *Foxo* vs. *D42*>*PI3K-CAAX*, p = 0.0041, 0.0005, 0.0002, 0.0006; vs. *D42*>*Foxo*, p = 0.50, 0.43, 0.16, 0.14. For $Foxo^{21}Foxo^{25}$; $OK6>PI3K^{DN}$ vs. $OK6>PI3K^{DN}$, p = ; 0.0003, 0.0004, 0.0014, 0.001. vs. $Foxo^{21}Foxo^{25}$, p = 0.63, 0.74, 0.43, 0.20. C) Mean ejp amplitude +/−SEMs (Y axis) for each genotype at the indicated [$Ca^{2+}$]. Nerves from six larvae were stimulated at a frequency of 1 Hz, and 10 responses were measured per larva. One-way ANOVA and Fisher's LSD gave the following differences, at 0.1 mM, 0.15 mM and 0.2 mM [$Ca^{2+}$], respectively: For *D42*>+ vs. $Foxo^{21}/Foxo^{25}$, p = 0.0079, <0.0001, 0.012; vs. *D42*>*Foxo*, p = 0.017, 0.0005, 0.10. For $Foxo^{21}/Foxo^{25}$; $OK6>PI3K^{DN}$: vs. $Foxo^{21}/Foxo^{25}$, p = 0.74, 0.12, 0.93; vs. $D42>PI3K^{DN}$, p<0.0001, <0.0001, 0.0001; vs. *D42*>*PTEN*, p<0.0001, <0.0001, <0.0001. For *D42*>*PI3K-CAAX*, *Foxo* vs. *D42*>*PI3K-CAAX*, p<0.0001, <0.0001, = 0.0024; vs. *D42*>*Foxo*, p = 0.52, 0.13, 0.77. D) Mean transmitter release success rate +/−SEMs (Y axis) at the indicated $Ca^{2+}$ concentration (X axis) for the indicated genotypes. Larval nerves were stimulated at 1 Hz. 10 responses were collected per nerve from each of 6 larvae for the given genotype and at the given $Ca^{2+}$ concentration. One-way ANOVA and Fisher's LSD gave the following differences, at 0.1 mM, 0.15 mM and 0.2 mM [$Ca^{2+}$], respectively: For *D42*>+ vs. $Foxo^{21}/Foxo^{25}$, p = 0.0008, 0.0039, 0.0009; vs. *D42*>*Foxo*, p = 0.0008, 0.004, 0.7. For $Foxo^{21}/Foxo^{25}$; $OK6>PI3K^{DN}$: vs. $Foxo^{21}/Foxo^{25}$, p = 0.81, 0.99, 0.43. vs. $D42>PI3K^{DN}$, p<0.0001, <0.0001, <0.0001. vs. *D42*>*PTEN*, p<0.0001, <0.0001. <0.0001. For *D42*>*PI3K-CAAX*, *Foxo* vs. *D42*>*PI3K-CAAX*, p<0.0001, <0.0001, = 0.002; vs. *D42*>*Foxo*, p = 0.29, 0.98, 0.7.
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$PI3K^{DN}$, *PTEN* or $Foxo^{+}$ alone are expressed in motor neurons. We confirmed this prediction: larvae co-expressing $Foxo^{+}$ and *PI3K-CAAX* in motor neurons exhibited rate of onset of LTF, basal transmitter release and failure frequency very similar to what was observed when $PI3K^{DN}$, *PTEN*, or $Foxo^{+}$ alone were expressed in motor neurons (Figure 3). Thus, eliminating *Foxo* reverses the hyperexcitability conferred by blocking PI3K pathway in motor neurons, whereas overexpressing $Foxo^{+}$ reverses the hypoexcitability confered by activating PI3K in motor neurons. These epistasis tests support the notion that PI3K activity decreases motor neuron excitability by inhibiting Foxo.

In contrast, we found that altering the Tor/S6K pathway had little effect on motor neuron excitability. In particular, motor neuron expression of neither the dominant-negative $S6K^{DN}$ nor the constitutively active $S6K^{Act}$ transgene [39] had any effect on the rate of onset of LTF (Figure 4). In addition, except for the appearance of some enhancement at the lowest stimulus frequency applied, expression of $S6K^{DN}$ had no effect on the ability of *PI3K-CAAX* to decrease the rate of onset of LTF (Figure 4). Furthermore, expression of $S6K^{DN}$ had no effect on basal transmitter release, and did not affect the ability of *PI3K-CAAX* to depress basal transmitter release (data not shown). Therefore we

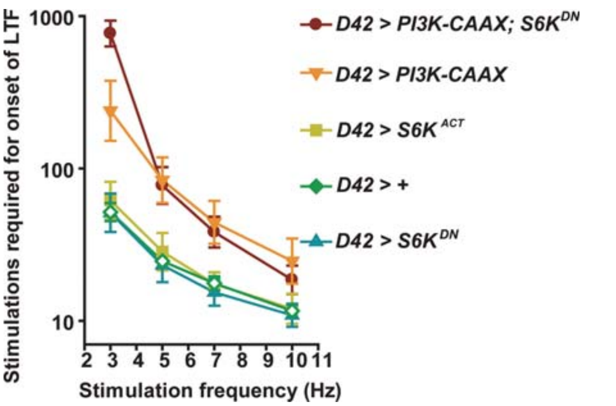


**Figure 4. *S6K* does not mediate the effects of *PI3K* on motor neuron excitability.** Number of stimulations required to induce LTF (Y axis) at the indicated stimulus frequencies (X axis). The bath solution contained 0.15 mM [$Ca^{2+}$] and 0.1 mM quinidine. Geometric means+/−SEMs are shown. From *top* to *bottom*, $n = 12$, 7, 9, 14, and 18 respectively, for each genotype.
doi:10.1371/journal.pgen.1000277.g004

conclude that the Tor/S6K pathway does not mediate the effects of PI3K on neuronal excitability.

### The Effects of PI3K on Synapse Number Are Mediated by Tor/S6 Kinase, Not Foxo

Because altered PI3K pathway activity alters motor neuron arborization and synapse number [26], it seemed possible that a causal relationship existed between the PI3K-mediated excitability and neuroanatomy defects. To test this possibility, we evaluated the roles of the Tor/S6K and Foxo pathways in mediating the effects of altered PI3K activity on synapse number. We found that motor neuron-specific expression of *S6K*$^{Act}$ increased synapse number to an extent similar to *PI3K-CAAX*, and motor neuron expression of *S6K*$^{DN}$ partially suppressed the increase in synapse number conferred by *PI3K-CAAX* (Figure 5A and 5B). These observations suggest that S6K mediates in part the effects of PI3K on arborization and synapse number. However, the ability of S6K$^{DN}$ to suppress only partially the effects of PI3K-CAAX overgrowth suggests that both Tor/S6K and a second, PI3K-mediated, pathway (presumably involving GSK3) regulate synapse formation. A role for the Tor/S6K in the control of synapse number was previously reported by Knox et al. (2007). In this report, null mutations in *S6K* decreased synapse number as well as muscle size at the larval nmj. However, it was further reported that activation of the PI3K effector Rheb, which activates Tor/S6K, increased synapse number at the larval nmj even when Tor activity was inhibited by rapamycin [40], raising the possibility that Rheb activates synapse formation via multiple redundant pathways, including Tor/S6K.

In contrast to the effects of altered S6K on synapse formation, we found that *Foxo*$^{+}$ overexpression had no effect on synapse number (data not shown) and failed to suppress the growth-promoting effects of PI3K-CAAX (Figure 5A and 5B).

We found that the PI3K pathway also affects axon diameter. In Drosophila peripheral nerves, about 80 axons, including about 35 motor axons, are wrapped by about three layers of glia, as shown in the transmission electron micrograph from cross sections of peripheral nerves in Figure 5C. We found that motor neuron specific expression of *PTEN* decreased motor axon diameter, whereas motor-neuron specific expression of *PI3K-CAAX* increased motor axon diameter. Tor/S6K, but not Foxo, mediates this growth effect. In particular, motor neuron-specific expression of *S6K*$^{Act}$ increased axon diameter to an extent similar to *PI3K-CAAX*, and motor-neuron-specific expression of *S6K*$^{DN}$ decreased motor axon diameter to an extent similar to *PTEN* and also partially suppressed the growth-promoting effects conferred by *PI3K-CAAX*. In contrast, *Foxo*$^{+}$ overexpression did not have a significant effect on the ability of PI3K-CAAX to increase axon diameter (Figure 5D). Therefore, Foxo mediates the excitability effects, but not the growth-promoting effects, of altered PI3K pathway activity, whereas the Tor/S6K pathway mediates in part the growth promoting effects but not the excitability effects of altered PI3K pathway. We conclude that the excitability and growth effects are completely separable genetically and thus have no causal relationship.

### Activity-Dependent Increase in Synapse Number Requires PI3K Activity

Depending on the system, neuronal activity can either restrict or promote synapse formation [41]. The Drosophila *eag Sh* double mutant, in which two distinct potassium channel subunits are simultaneously disrupted, displays extreme neuronal hyperexcitability [25], and a consequent increase in synapse number [42,43]. This activity-dependent increase in synapse number does not require DmGluRA activity [16], suggesting that excessive glutamate release is not necessary for this excessive growth to occur. To determine if PI3K activity is required for this overgrowth, we compared synapse number in wildtype larvae, in larvae expressing dominant-negative transgenes for both *eag* (*eag*$^{DN}$) and *Sh* (*Sh*$^{DN}$) [44,45] in motor neurons, and in larvae co-expressing *eag*$^{DN}$, *Sh*$^{DN}$ and *PI3K*$^{DN}$. We found that co-expression of *eag*$^{DN}$ and *Sh*$^{DN}$ in motor neurons increased synapse number similarly to what was observed previously [42], and that this increase was completely blocked by simultaneous expression of *PI3K*$^{DN}$ but not by *lacZ* (Figure 6). Thus, the activity-dependent increase in synapse formation requires PI3K activity. The observation that glutamate activation of DmGluRA is not necessary for this increase raises the possibility that another PI3K activator contributes to synapse formation at the larval nmj. Insulin is a plausible candidate for such an activator because both insulin and insulin receptor immunoreactivity are present at the nmj [46].

## Discussion

### A Mechanism for the Glutamate-Induced Negative Feedback of Motor Neuron Excitability

The effects on neuronal excitability of altered DmGluRA, PI3K, and Foxo activities are consistent with a model in which glutamate released from motor nerve terminals as a consequence of motor neuron activity activates motor neuron PI3K via DmGluRA autoreceptors, which then downregulate neuronal excitability via inhibition of Foxo (Figure 7). Foxo, in turn, might regulate excitability via transcription of ion channel subunits or regulators. Although such putative Foxo targets have not been identified, one potential target might be the translational repressor encoded by *pumilio (pum)*: *pum* expression is downregulated by neuronal activity, Pum decreases transcript levels of the sodium channel encoded by *para*, and both *para* overexpression and *pum* mutations increase rate of onset of LTF in a manner similar to that described here [19,21,23,24].

### Other Negative Feedback Systems at the Drosophila nmj

The DmGluRA-dependent negative feedback reported here co-exists with several other negative feedback systems operating at the

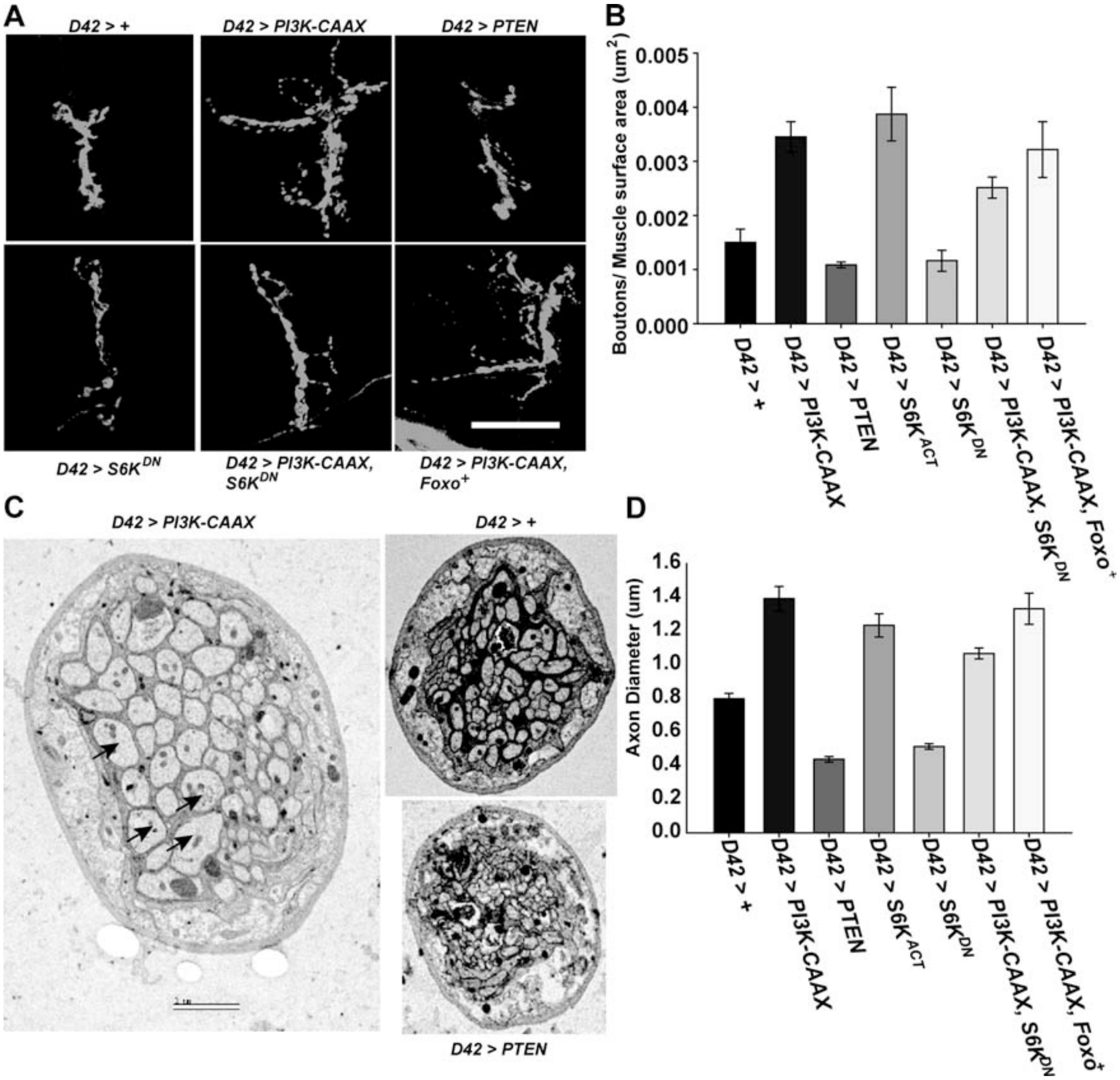


**Figure 5. PI3K regulates synapse formation and axon growth via S6K, not Foxo.** A) Representative images of muscles 7 and 6 in the indicated genotypes. Larva were stained with anti-HRP (green). Scale bar = 50 µm. B) Mean number (+/−S.E.M.s) of synaptic boutons normalized to the surface area of muscle 6 at abdominal segment A3 in the indicated genotypes. From left to right, n = 6, 8, 6, 6, 7, 11, 11, respectively, for each genotype. One-way ANOVA and Fisher's LSD gave the following differences: For *D42*>*S6K*$^{Act}$ vs. *D42*>*PI3K-CAAX*, p = 0.40; vs. *D42*>+, p = 0.0009. For *D42*>*PI3K-CAAX* vs. *D42*>*PI3K-CAAX*, *Foxo*, p = 0.64; vs. *D42*>*PI3K-CAAX*, *S6K*$^{DN}$, p = 0.05. C) Representative transmission electron micrographs of peripheral nerve cross sections. Axons are marked with arrows. Scale bar = 2 µm. D) Mean axon diameter (+/−S.E.M.s) of the five largest axons from five different nerves (25 measurements total) for the indicated genotypes. One-way ANOVA and Fisher's LSD gave the following significant differences: for *D42*>+: vs. *D42*>*PI3K-CAAX*, p<0.0001; vs, *D42*>*PTEN*, p<0.0001; vs. *D42*>*S6K*$^{DN}$, p = 0.0009; vs. *D42*>*S6K*$^{act}$, p<0.0001; vs. *D42*>*PI3K-CAAX*, *Foxo*, p<0.0001; vs *D42*>*PI3K-CAAX*, *S6K*$^{DN}$, p = 0.0011. For *D42*>*PI3K-CAAX* vs. *D42*>*PI3K-CAAX*, *S6K*$^{DN}$, p = 0.0002. Means from *D42*>*PI3K-CAAX* and *D42*>*PI3K-CAAX*, *Foxo* were judged to be not significantly different (p = 0.43).
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Drosophila nmj. In addition to altered excitability, these systems include alterations in the vesicle release properties of the motor nerve terminal and density of the muscle glutamate receptors [1]. Presumably, these diverse feedback systems, acting in parallel, regulate specific aspects of neuronal function. The DmGluRA-dependent feedback system reported here differs in several respects from some of the other feedback systems reported. For example, this DmGluRA-dependent feedback apparently involves transcriptional changes, suggesting that this system operates on a long time scale and thus will be responsive to chronic, rather than acute, changes in neuronal activity. In addition, this system is likely to be motor neuron-cell autonomous, and will not involve participation of additional cells, such as target muscles or adjacent glia. Furthermore, this system is predicted to link mechanistically several PI3K-dependent processes, including activity-dependent downregulation of neuronal excitability and upregulation of neuronal growth. In this regard, the PI3K-dependent inhibition of Foxo might protect neurons from excitotoxic effects of prolonged stimulation; such protection would not be accomplished by the other feedback systems operating.

## Role of Mammalian mGluRs and PI3K in Regulation of Ion Channel Activity

Both mGluRs and PI3K have been previously implicated in regulation of ion channel activity. For example, ligand activation of group I mGluRs trigger Gαq-mediated release of $Ca^{2+}$ from intracellular stores, and consequently activate $Ca^{2+}$-dependent $K^{+}$ channels and nonselective cation channels [47], whereas activation

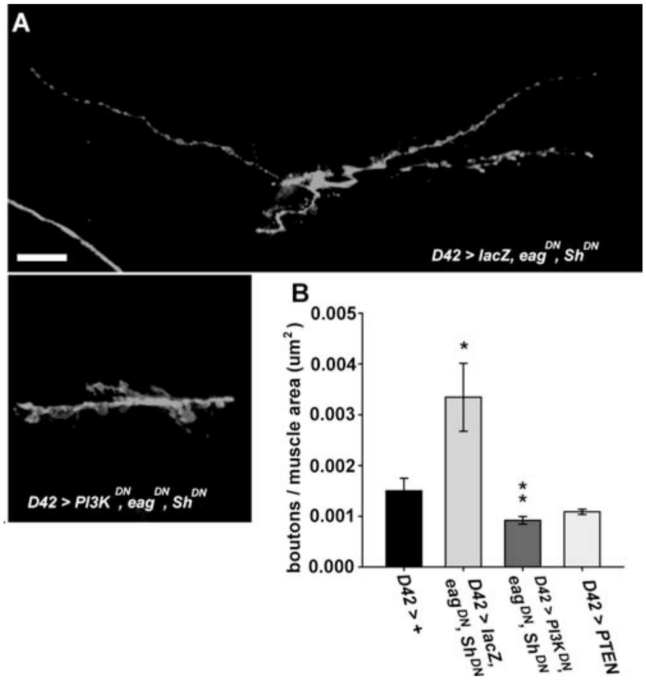


**Figure 6. *PI3K$^{DN}$* expression suppresses the synaptic overgrowth conferred by motor neuron expression of *eag$^{DN}$* and *Sh$^{DN}$*.** The *D42 Gal4* driver was used to induce motor neuron transgene expression. A) Representative confocal images of muscles 7 and 6 in the indicated genotypes. Larvae were stained with anti-HRP (green). Scale bar = 20 µm. B) Mean number (+/−S.E.M.s) of synaptic boutons normalized to the surface area (Y axis) of muscle 6 at abdominal segment A3 in the indicated genotypes (X axis). From *top* to *bottom*, *n* = 12, 6, 6 and 6 respectively, for each genotype. One-way ANOVA and Fisher's LSD gave the following differences: For *D42>lacZ*, *eag$^{DN}$*, *Sh$^{DN}$* vs. *D42>+*, p = 0.027; vs. *D42>PI3K$^{DN}$*, *eag$^{DN}$*, *Sh$^{DN}$*, p = 0.0075. For *D42>PI3K$^{DN}$*, *eag$^{DN}$*, *Sh$^{DN}$* vs. *D42>PTEN*, p = 0.86.
doi:10.1371/journal.pgen.1000277.g006

of group II mGluRs inhibit transmitter release via inhibition of P/Q $Ca^{2+}$ channels [48,49]. PI3K activation can promote ion channel insertion into cell membranes [50–52] and can mediate the decrease in excitability conferred by application of leptin, the product of the obese gene, by activating $Ca^{2+}$-dependent $K^+$ channels [53]. However, to our knowledge, effects of mGluR or PI3K activation on ion channel transcription in the nervous system have not been reported.

PI3K also regulates ion channel activity in non-excitable cells. PI3K mediates the ability of insulin growth factor to activate the Eag channel, and the ability of serum to activate the intermediate-conductance $Ca^{2+}$-activated $K^+$ channel in breast carcinoma and lymphoma cells, respectively [54,55]. Interestingly, in these non-excitable cells, activation is accomplished by both acute effects on channel activity as well as long term effects as a consequence of increased channel transcription. Therefore, PI3K can regulate channel activity over different time courses, and via distinct mechanisms, presumably via distinct effector pathways.

## Neuronal Excitability in Human Disease

Human orthologues of group II mGluRs and PI3K are implicated in several neurological disorders. For example, group II mGluRs are potential drug targets for schizophrenia, epilepsy, and anxiety disorders [12–14], raising the possibility that altered excitability of glutamatergic neurons might play a role in these disorders. In addition, levels of phospho-Foxo, a product of PI3K/

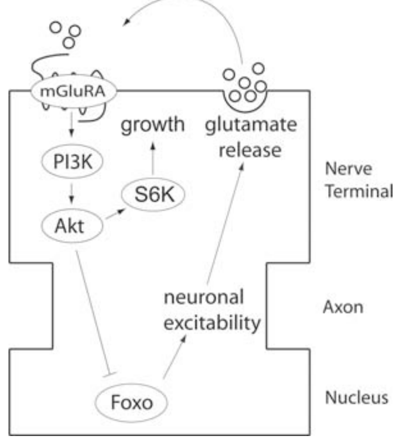


**Figure 7. A model for the negative feedback loop regulating motor neuron excitability.** The transcription factor Foxo increases neuronal excitability through a mechanism possibly involving transcription of ion channel subunits or regulators. This increased excitability promotes glutamate release from motor nerve terminals, which then activates presynaptic DmGluRA in an autocrine manner. This activation, in turn, activates PI3K and the subsequent inactivation of Foxo by Akt-mediated inhibitory phosphorylation. Activated PI3K also promotes axonal growth and synapse formation via the Tor/S6K pathway.
doi:10.1371/journal.pgen.1000277.g007

Akt activity, are increased following induction of seizures in rats, and in the hippocampi of epileptic patients [56]. This activity-induced increase in phospho-Foxo was interpreted as a mechanism to protect neurons from the excitotoxic effects of excessive glutamate release because Foxo is more likely than phospho-Foxo to promote apoptosis. Our results raise the possibility that this increase in phospho-Foxo levels occurs via glutamate-induced PI3K activation mediated by group II mGluRs, and interpret this increase as a negative feedback on excitability. A role for PI3K activity in inhibiting epileptic seizures is further supported by the recent observation that application of leptin, a known PI3K activator, inhibits seizures in a PI3K-dependent manner [57]. Increased insulin/IGF levels and increased PI3K activity are also implicated in autism spectrum disorders [58,59]. These increases are generally hypothesized to affect neuronal function by increasing arborization and synapse formation, but our results raise the possibility that altered neuronal excitability might also contribute. Thus, the results reported here might have significance for several human neurological disorders.

## A Novel Signalling Pathway Linking Group II mGluRs and PI3K

The mechanism by which glutamate-activated DmGluRA activates PI3K remains unknown. Although mammalian group I mGluRs activate PI3K via the Homer scaffolding protein and the PI3K enhancer PIKE [60], Drosophila mGluRA, similar to mammalian group II mGluRs, lack Homer binding motifs [61] and thus would not be predicted to activate PI3K by this mechanism. Alternatively, although the inhibition of glutamate-induced p-Akt activation by *PI3K$^{DN}$* expression demonstrates that PI3K activity is required for this activation, it remains possible that glutamate increases p-Akt levels by activating an enzyme in addition to PI3K. For example, Akt is reported to be phosphorylated and activated by Calmodulin-dependent kinase kinase [62]. Additionally, glutamate-activated DmGluRA might activate PI3K in motor nerve terminals indirectly by triggering $Ca^{2+}$ release from stores, leading to release of insulin and hence activation of PI3K by well-established mechanisms. Further experiments will be required to address these issues.

## Materials and Methods

### Drosophila Stocks

Fly stocks were maintained on standard cornmeal/ agar Drosophila media at room temperature. *D42* and *OK6* express *Gal4* in motor neurons and were provided by Tom Schwarz, Boston, Massachusetts, and Hermann Aberle, Tubingen, Germany respectively. The *UAS-PI3K*$^{DN}$ (D954A) and *UAS-PI3K-CAAX* transgenes were provided by Sally Leevers, London, UK, the *UAS-Foxo*$^{+}$ transgene was provided by Marc Tatar, Providence, RI, the *Foxo*$^{21}$ and *Foxo*$^{25}$ lines were provided by Heinrich Jasper, Rochester, NY, the *UAS-S6K*$^{DN}$ and *UAS-S6K*$^{act}$ transgenes were provided by Ping Shen, Athens, GA, and the *UAS-DmGluRA-RNAi* and the *DmGluRA*$^{112b}$ lines were provided by Marie-Laure Parmentier, Montpelier, France. All other fly stocks were provided by the Drosophila stock center, Bloomington, IN.

### Immunocytochemistry

FITC conjugated antibodies against horseradish peroxidase (HRP) were raised in goat (Jackson ImmunoResearch) and were used at 1:400 dilution. Antibodies against Drosophila p-Akt (Ser505) were raised in rabbit (Cell Signaling Technologies) and were used at 1:500 dilution. Rhodamine Red conjugated goat anti-rabbit (Jackson ImmunoResearch) was used at a dilution of 1:1000. For arborization measurements, larvae were dissected in PBS-T and fixed in 4% paraformaldehyde. Images were taken on a Zeiss 410 laser scanning confocal microscope (LSM) with a 20× objective. ImageJ was used to obtain surface area measurements of muscle 6 from abdominal segment A3, and the number of boutons was counted manually. For p-Akt measurements, larvae were dissected in Grace's insect cell culture media (Gibco). When glutamate was applied,100 μM glutamic acid monosodium salt monohydrate (Acros Organics) dissolved in Grace's insect cell culture media was added to the well of the dissection plate. 1 minute after glutamate addition, larvae were rapidly washed in standard saline (0.128 M NaCl, 2.0 mM KCl, 4.0 mM $MgCl_2$, 0.34 M sucrose, 5.0 mM HEPES, pH 7.1, and 0.15 mM $CaCl_2$), and then immediately fixed in 4% paraformaldehyde. For the 10 min wash, the larvae were washed in Grace's insect cell culture media and placed on shaker for 10 minutes before fixing. Care was taken to treat all samples identically during this procedure. Images were taken on a Zeiss 510 LSM with a 20× objective. Z-stacks were compiled from 2 μm serial sections to a depth adequate to encompass the entire bouton thickness for each sample (from 8–20 μm). Muscles 7 and 6 from either abdominal segments A3 or A4 were used for measurements. ImageJ software was used to analyze p-Akt intensities. In particular, 2D projections were created using the median pixel intensity from each stack at each coordinate point. Neuronal structures, marked by anti-HRP, were traced using the freehand selection tool and the selection was transferred to the anti-p-Akt image where the mean pixel intensity value was measured. Background was obtained with a selection box encompassing the non-neuronal area of muscles 6 and 7 in the particular abdominal segment, the mean pixel intensity was measured and subtracted from the mean p-Akt pixel intensity.

### Electrophysiology

The *D42* motor neuron driver was used to express transgenes for all experiments, except that the *OK6* driver was used for experiments the *Foxo*$^{21}$/*Foxo*$^{25}$ genotype was included. *OK6* is located on a different chromosome from *Foxo*, which simplifies stock construction. Larvae were grown to the wandering third-instar stage in uncrowded bottles at room temperature and dissected as described (17, 18) in standard saline solution (128 mM NaCl, 2.0 mM KCl, 4.0 mM $MgCl_2$, 34 mM sucrose, 5.0 mM HEPES, pH 7.1, and $CaCl_2$ as specified in the text). Peripheral nerves were cut posterior to the ventral ganglion and were stimulated using a suction electrode. Muscle recordings were taken from muscle 6 in abdominal sections 3–5. Stimulation intensity (5 V for approximately 0.05 msec) was adjusted to 1.5 times threshold, which reproducibly stimulates both axons innervating muscle cell 6. Recording electrodes were pulled using a Flaming/ Brown micropipette puller to a tip resistance of 10–40 MΩ and filled with 3M KCl. LTF and ejp amplitude data are reported as geometric, rather than arithmetic means, because the data show a positive skew. For extracellular recordings of neuronal action potentials, a loop of nerve near the nerve terminal was introduced into a suction electrode and nerve activity recorded with a DAM-80 differential amplifier.

### Electron Microscopy

Larvae were grown to the wandering third-instar stage in uncrowded bottles at room temperature. Dissections and preparation for microscopy were performed as previously described [63]. Nerve cross sections close to (within about 10 μm from) the ventral ganglion were obtained and analyzed. Axon diameter measurements were taken from the five largest axons from five different nerves from at least two different larvae.

## Supporting Information

**Figure S1** LTF onset is accompanied by supernumerary action potentials in the peripheral nerve. Simultaneous intracellular recordings from muscle (lower traces) and extracellular recordings from the innervating peripheral nerve (upper traces) in the indicated genotypes in response to 10 Hz nerve stimulation. Responses are shown immediately prior to and immediately following LTF onset. Note that LTF onset in each genotype, indicated by arrows, was accompanied by supernumerary, repetitive firing of axons in the innervating nerve (arrowheads). Bath [$Ca^{2+}$] was 0.15 mM, quinidine concentration was 0.1 mM.
Found at: doi:10.1371/journal.pgen.1000277.s001 (6.7 MB TIF)

**Figure S2** PI3K pathway inhibition increases neuronal excitability. Number of stimulations required to induce LTF (Y axis) at the indicated stimulus frequencies (X axis) in the indicated genotypes. Geometric means+/−SEMs are shown. Bath [$Ca^{2+}$] was 0.15 mM. n = 5 for all genotypes. One-way ANOVA and Fisher's LSD gave the following differences, at 10 Hz, 7 Hz, 5 Hz respectively: For *D42*>+: vs. *D42*>*PI3K*$^{DN}$, p = 0.027, 0.020, 0.0033; vs. *D42*>Foxo, p = 0.0099, 0.018, <0.0001.
Found at: doi:10.1371/journal.pgen.1000277.s002 (7.0 MB TIF)

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## Author Contributions

Conceived and designed the experiments: EH MS. Performed the experiments: EH CCJL WL. Analyzed the data: EH. Contributed reagents/materials/analysis tools: MS. Wrote the paper: EH MS.

## References


1. Davis GW (2006) Homeostatic Control of Neural Activity: From Phenomenology to Molecular Design. Annu Rev Neurosci.
2. Marder E, Prinz AA (2003) Current compensation in neuronal homeostasis. Neuron 37: 2–4.
3. Pozzi D, Condliffe S, Bozzi Y, Chikhladze M, Grumelli C, et al. (2008) Activity-dependent phosphorylation of Ser187 is required for SNAP-25-negative modulation of neuronal voltage-gated calcium channels. Proc Natl Acad Sci U S A 105: 323–328.
4. Desai NS, Rutherford LC, Turrigiano GG (1999) Plasticity in the intrinsic excitability of cortical pyramidal neurons. Nat Neurosci 2: 515–520.
5. Echegoyen J, Neu A, Graber KD, Soltesz I (2007) Homeostatic plasticity studied using in vivo hippocampal activity-blockade: synaptic scaling, intrinsic plasticity and age-dependence. PLoS ONE 2: e700.
6. Gibson JR, Bartley AF, Huber KM (2006) Role for the subthreshold currents ILeak and IH in the homeostatic control of excitability in neocortical somatostatin-positive inhibitory neurons. J Neurophysiol 96: 420–432.
7. Chen CY, Ling Eh EH, Horowitz JM, Bonham AC (2002) Synaptic transmission in nucleus tractus solitarius is depressed by Group II and III but not Group I presynaptic metabotropic glutamate receptors in rats. J Physiol 538: 773–786.
8. Kew JN, Ducarre JM, Pflimlin MC, Mutel V, Kemp JA (2001) Activity-dependent presynaptic autoinhibition by group II metabotropic glutamate receptors at the perforant path inputs to the dentate gyrus and CA1. Neuropharmacology 40: 20–27.
9. Poisik O, Raju DV, Verreault M, Rodriguez A, Abeniyi OA, et al. (2005) Metabotropic glutamate receptor 2 modulates excitatory synaptic transmission in the rat globus pallidus. Neuropharmacology 49 Suppl 1: 57–69.
10. Scanziani M, Salin PA, Vogt KE, Malenka RC, Nicoll RA (1997) Use-dependent increases in glutamate concentration activate presynaptic metabotropic glutamate receptors. Nature 385: 630–634.
11. Schoepp DD (2001) Unveiling the functions of presynaptic metabotropic glutamate receptors in the central nervous system. J Pharmacol Exp Ther 299: 12–20.
12. Patil ST, Zhang L, Martenyi F, Lowe SL, Jackson KA, et al. (2007) Activation of mGlu2/3 receptors as a new approach to treat schizophrenia: a randomized Phase 2 clinical trial. Nat Med 13: 1102–1107.
13. Swanson CJ, Bures M, Johnson MP, Linden AM, Monn JA, et al. (2005) Metabotropic glutamate receptors as novel targets for anxiety and stress disorders. Nat Rev Drug Discov 4: 131–144.
14. Alexander GM, Godwin DW (2006) Metabotropic glutamate receptors as a strategic target for the treatment of epilepsy. Epilepsy Res 71: 1–22.
15. Anwyl R (1999) Metabotropic glutamate receptors: electrophysiological properties and role in plasticity. Brain Res Brain Res Rev 29: 83–120.
16. Bogdanik L, Mohrmann R, Ramaekers A, Bockaert J, Grau Y, et al. (2004) The Drosophila metabotropic glutamate receptor DmGluRA regulates activity-dependent synaptic facilitation and fine synaptic morphology. J Neurosci 24: 9105–9116.
17. Jan YN, Jan LY (1978) Genetic dissection of short-term and long-term facilitation at the Drosophila neuromuscular junction. Proc Natl Acad Sci U S A 75: 515–519.
18. Stern M, Ganetzky B (1989) Altered synaptic transmission in Drosophila hyperkinetic mutants. J Neurogenet 5: 215–228.
19. Stern M, Kreber R, Ganetzky B (1990) Dosage effects of a Drosophila sodium channel gene on behavior and axonal excitability. Genetics 124: 133–143.
20. Chouinard SW, Wilson GF, Schlimgen AK, Ganetzky B (1995) A potassium channel beta subunit related to the aldo-keto reductase superfamily is encoded by the Drosophila hyperkinetic locus. Proc Natl Acad Sci U S A 92: 6763–6767.
21. Loughney K, Kreber R, Ganetzky B (1989) Molecular analysis of the para locus, a sodium channel gene in Drosophila. Cell 58: 1143–1154.
22. Mallart A, Angaut-Petit D, Bourret-Poulain C, Ferrus A (1991) Nerve terminal excitability and neuromuscular transmission in T(X;Y)V7 and Shaker mutants of Drosophila melanogaster. J Neurogenet 7: 75–84.
23. Mee CJ, Pym EC, Moffat KG, Baines RA (2004) Regulation of neuronal excitability through pumilio-dependent control of a sodium channel gene. J Neurosci 24: 8695–8703.
24. Schweers BA, Walters KJ, Stern M (2002) The Drosophila melanogaster translational repressor pumilio regulates neuronal excitability. Genetics 161: 1177–1185.
25. Ganetzky B, Wu CF (1982) Drosophila mutants with opposing effects on nerve excitability: genetic and spatial interactions in repetitive firing. J Neurophysiol 47: 501–514.
26. Martin-Pena A, Acebes A, Rodriguez JR, Sorribes A, de Polavieja GG, et al. (2006) Age-independent synaptogenesis by phosphoinositide 3 kinase. J Neurosci 26: 10199–10208.
27. Brand AH, Perrimon N (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118: 401–415.
28. Parkes TL, Elia AJ, Dickinson D, Hilliker AJ, Phillips JP, et al. (1998) Extension of Drosophila lifespan by overexpression of human SOD1 in motorneurons. Nat Genet 19: 171–174.
29. Leevers SJ, Weinkove D, MacDougall LK, Hafen E, Waterfield MD (1996) The Drosophila phosphoinositide 3-kinase Dp110 promotes cell growth. Embo J 15: 6584–6594.
30. Huang Y, Stern M (2002) In vivo properties of the Drosophila inebriated-encoded neurotransmitter transporter. J Neurosci 22: 1698–1708.
31. Dionne MS, Pham LN, Shirasu-Hiza M, Schneider DS (2006) Akt and FOXO dysregulation contribute to infection-induced wasting in Drosophila. Curr Biol 16: 1977–1985.
32. Colombani J, Bianchini L, Layalle S, Pondeville E, Dauphin-Villemant C, et al. (2005) Antagonistic actions of ecdysone and insulins determine final size in Drosophila. Science 310: 667–670.
33. Palomero T, Sulis ML, Cortina M, Real PJ, Barnes K, et al. (2007) Mutational loss of PTEN induces resistance to NOTCH1 inhibition in T-cell leukemia. Nat Med 13: 1203–1210.
34. Hay N, Sonenberg N (2004) Upstream and downstream of mTOR. Genes Dev 18: 1926–1945.
35. Tang ED, Nunez G, Barr FG, Guan KL (1999) Negative regulation of the forkhead transcription factor FKHR by Akt. J Biol Chem 274: 16741–16746.
36. Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA (1995) Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature 378: 785–789.
37. Junger MA, Rintelen F, Stocker H, Wasserman JD, Vegh M, et al. (2003) The Drosophila forkhead transcription factor FOXO mediates the reduction in cell number associated with reduced insulin signaling. J Biol 2: 20.
38. Hwangbo DS, Gershman B, Tu MP, Palmer M, Tatar M (2004) Drosophila dFOXO controls lifespan and regulates insulin signalling in brain and fat body. Nature 429: 562–566.
39. Barcelo H, Stewart MJ (2002) Altering Drosophila S6 kinase activity is consistent with a role for S6 kinase in growth. Genesis 34: 83–85.
40. Knox S, Ge H, Dimitroff BD, Ren Y, Howe KA, et al. (2007) Mechanisms of TSC-mediated control of synapse assembly and axon guidance. PLoS ONE 2: e375.
41. Vicario-Abejon C, Owens D, McKay R, Segal M (2002) Role of neurotrophins in central synapse formation and stabilization. Nat Rev Neurosci 3: 965–974.
42. Budnik V, Zhong Y, Wu CF (1990) Morphological plasticity of motor axons in Drosophila mutants with altered excitability. J Neurosci 10: 3754–3768.
43. Davis GW, Schuster CM, Goodman CS (1996) Genetic dissection of structural and functional components of synaptic plasticity. III. CREB is necessary for presynaptic functional plasticity. Neuron 17: 669–679.
44. Broughton SJ, Kitamoto T, Greenspan RJ (2004) Excitatory and inhibitory switches for courtship in the brain of Drosophila melanogaster. Curr Biol 14: 538–547.
45. Mosca TJ, Carrillo RA, White BH, Keshishian H (2005) Dissection of synaptic excitability phenotypes by using a dominant-negative Shaker K+ channel subunit. Proc Natl Acad Sci U S A 102: 3477–3482.
46. Gorczyca M, Augart C, Budnik V (1993) Insulin-like receptor and insulin-like peptide are localized at neuromuscular junctions in Drosophila. J Neurosci 13: 3692–3704.
47. Fagni L, Chavis P, Ango F, Bockaert J (2000) Complex interactions between mGluRs, intracellular Ca2+ stores and ion channels in neurons. Trends Neurosci 23: 80–88.
48. Mela F, Marti M, Fiorentini C, Missale C, Morari M (2006) Group-II metabotropic glutamate receptors negatively modulate NMDA transmission at striatal cholinergic terminals: role of P/Q-type high voltage activated Ca++ channels and endogenous dopamine. Mol Cell Neurosci 31: 284–292.
49. Robbe D, Bockaert J, Manzoni OJ (2002) Metabotropic glutamate receptor 2/3-dependent long-term depression in the nucleus accumbens is blocked in morphine withdrawn mice. Eur J Neurosci 16: 2231–2235.
50. Dryer SE, Lhuillier L, Cameron JS, Martin-Caraballo M (2003) Expression of K(Ca) channels in identified populations of developing vertebrate neurons: role of neurotrophic factors and activity. J Physiol Paris 97: 49–58.
51. Hou Q, Zhang D, Jarzylo L, Huganir RL, Man HY (2008) Homeostatic regulation of AMPA receptor expression at single hippocampal synapses. Proc Natl Acad Sci U S A 105: 775–780.
52. Viard P, Butcher AJ, Halet G, Davies A, Nurnberg B, et al. (2004) PI3K promotes voltage-dependent calcium channel trafficking to the plasma membrane. Nat Neurosci 7: 939–946.
53. Shanley LJ, O'Malley D, Irving AJ, Ashford ML, Harvey J (2002) Leptin inhibits epileptiform-like activity in rat hippocampal neurones via PI 3-kinase-driven activation of BK channels. J Physiol 545: 933–944.
54. Borowiec AS, Hague F, Harir N, Guenin S, Guerineau F, et al. (2007) IGF-1 activates hEAG K(+) channels through an Akt-dependent signaling pathway in breast cancer cells: role in cell proliferation. J Cell Physiol 212: 690–701.
55. Wang J, Xu YQ, Liang YY, Gongora R, Warnock DG, et al. (2007) An intermediate-conductance Ca(2+)-activated K (+) channel mediates B lymphoma cell cycle progression induced by serum. Pflugers Arch 454: 945–956.
56. Shinoda S, Schindler CK, Meller R, So NK, Araki T, et al. (2004) Bim regulation may determine hippocampal vulnerability after injurious seizures and in temporal lobe epilepsy. J Clin Invest 113: 1059–1068.
57. Xu L, Rensing N, Yang XF, Zhang HX, Thio LL, et al. (2008) Leptin inhibits 4-aminopyridine- and pentylenetetrazole-induced seizures and AMPAR-mediated synaptic transmission in rodents. J Clin Invest 118: 272–280.

58. Kwon CH, Luikart BW, Powell CM, Zhou J, Matheny SA, et al. (2006) Pten regulates neuronal arborization and social interaction in mice. Neuron 50: 377–388.
59. Mills JL, Hediger ML, Molloy CA, Chrousos GP, Manning-Courtney P, et al. (2007) Elevated levels of growth-related hormones in autism and autism spectrum disorder. Clin Endocrinol (Oxf) 67: 230–237.
60. Rong R, Ahn JY, Huang H, Nagata E, Kalman D, et al. (2003) PI3 kinase enhancer-Homer complex couples mGluRI to PI3 kinase, preventing neuronal apoptosis. Nat Neurosci 6: 1153–1161.
61. Diagana TT, Thomas U, Prokopenko SN, Xiao B, Worley PF, et al. (2002) Mutation of Drosophila homer disrupts control of locomotor activity and behavioral plasticity. J Neurosci 22: 428–436.
62. Yano S, Tokumitsu H, Soderling TR (1998) Calcium promotes cell survival through CaM-K kinase activation of the protein-kinase-B pathway. Nature 396: 584–587.
63. Yager J, Richards S, Hekmat-Scafe DS, Hurd DD, Sundaresan V, et al. (2001) Control of Drosophila perineurial glial growth by interacting neurotransmitter-mediated signaling pathways. Proc Natl Acad Sci U S A 98: 10445–10450.